EXTRA CHROMOSOMAL DNA DIVERSITY AND DRUG RESISTANCE IN *AEROMONAS HYDROPHILA* OF AQUACULTURE SYSTEMS

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CERTIFICATE

Certified that the dissertation entitled **"EXTRA CHROMOSOMAL DNA DIVERSITY AND DRUG RESISTANCE IN *AEROMONAS HYDROPHILA* OF AQUACULTURE SYSTEMS"** is a record of independent bonafide research work carried out by **BRIJESH KUMAR HALAWAI (MC-78)** during the period of study from September 2001 to August 2003 under our supervision and guidance for the degree of **Master of Fisheries Science (Mariculture)** at the Central Marine Fisheries Research Institute, Kochi, and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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I, hereby declare that the thesis entitled **"EXTRA CHROMOSOMAL DNA DIVERSITY AND DRUG RESISTANCE IN *AEROMONAS HYDROPHILA* OF AQUACULTURE SYSTEMS"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

30 JUNE, 2003
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This work is

Dedicated to my Beloved Parents

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सारांश

मछली, जल और तलछट नमूनों से संग्रहित बाईस *ऐरोमोनास हइड्रोफिला* वियुक्तों को, उनके प्लैज़मिड, डी एन ए, प्रोटीन प्रोफाइल और इसके साथ साथ बीस प्रतिसूक्ष्मजीवी एजेंटों की ओर इनकी सुप्रभाव्यता जानने के लिए स्क्रीन किया गया । प्लैस्मिड उपस्थिति शत प्रतिशत था और 21 किलबेस प्लैस्मिड सभी वियुक्तों के लिए समान था । प्लैस्मिडों की संख्या 1 से 4 के बीच में थी तो आकार 1 से 21 किलोबेस के बीच था । इन वियुक्तों के बीच गुणसूत्रबाह्य डी एन ए की वैविद्यता विभिन्न प्रकार के आठ प्लैस्मिड प्राफाइलों से प्रतिबिम्बित थी । वियुक्तों में पचास प्रतिशत द्वारा प्रदर्शित प्रोफाइलों में अधिकतर 21 किलोबेस के एकल पट्टी थे । सभी वियुक्तों ने ग्यारह से चौदह प्रतिजैविकों पर बहुविध प्रतिरोध प्रतिरूप दिखाया । सभी वियुक्त ऐम्बिसिलिन, एमोक्सिलिन, सेफालेक्सिन, वांकोमाइसिन, बासिट्रासिन, पेनसिलिन-जी और लिंकोमाइसिन की ओर प्रतिरोधी थे, जब कि सभी ऑफ्लाक्सिन, नाइट्रोफ्यूराज़ोन, जेन्टामाइसिन, क्लोराम्फेनिकोल और पेफलोक्सिन की ओर संवेदनशील थे । **नेटीव-पेज** और **एस डी एस-पेज** के माध्यम से विघटित कोशिकीय प्रोटीन प्रोफाइल भी वियुक्तों के बीच विषमांगता व्यक्त की । कुछ प्रोटीन पट्टी सभी वियुक्तों में थे तो कुछ सभी वियुक्तों में नहीं थे । आर.ए.पी.डी प्रोफाइल उत्पप्प करने के लिए विभिन्न प्लैज़मिड प्रोफाइल वर्गों से यदृच्छिक प्रारंभकों के प्रयोग करके पी सी आर वर्धन भी किया गया । सभी वियुक्तों द्वारा उत्पादित कुछ प्रवर्धक जाति विशिष्ट थे तो अन्य भेदमूलक थे । आर. ए. पी. डी विश्लेषण वियुक्तों के बीच प्लैज़मिड डी एन ए वैविद्यता का उच्च स्तर स्पष्ट किया जो एकल प्लैज़मिड प्राफाइलन में स्पष्ट नहीं था, और इस प्रकार *ए.हाइड्रोफिला* के गुणसूत्रबाह्य वैविद्यता और जानपदिक रोगविज्ञानीय अध्ययन के एक अच्छा उपकरण बन जाता है । जलकृषि प्रणाली से *ए. हाइड्रोफिला* के गुणसूत्रबाह्य डी एन ए की अंतराजातीय वैविद्यता प्लैज़मिड प्रोफाइल, एन्टिबयोग्राम, कोशिकीय प्रोटीन प्रोफाइल और डी एन ए के आर ए पी डी प्रतिरूप से स्पष्ट थे ।

ABSTRACT

Twenty-two isolates of *Aeromonas hydrophila* collected from fish, water and sediment samples of aquaculture systems were screened for their plasmid DNA and protein profiles as well as susceptibility to twenty antimicrobial agents. The plasmid occurrence rate was 100% and a 21 Kilo base plasmid was common to all isolates. The number of plasmid ranged from 1 to 4 while size ranged from 1 to 21 Kilo base. The eight different types of plasmid profiles among these isolates reflected diversity of extrachromosomal DNA. The most common plasmid profile was a single banded of 21 Kilo base exhibited by 50% of the isolates. All the isolates showed multiple resistance patterns ranging from resistance to eleven to fourteen antibiotics. All the isolates were resistant to ampicillin, amoxycillin, cephalexin, vancomycin, bacitracin, penicillin-G and lincomycin, while all were sensitive to ofloxacin, nitrofurazone, gentamycin, chloramphenicol and pefloxacin. Cellular protein profile resolved through NATIVE-PAGE and SDS-PAGE also revealed heterogeneity among the isolates. While some of the protein bands were shared by all isolates, others were varied in the isolates. Random primed PCR amplification of the plasmid DNA from different plasmid profile groups was also carried out to generate RAPD profile. While some of the amplicons were species specific, produced by all the isolates, others were discriminatory. The RAPD analysis revealed high level of plasmid DNA diversity among the isolates, which were not evident in simple plasmid profiling, making it good tool for evaluation of extrachromosomal diversity and epidemiological studies of *A. hydrophila*. Intraspecies diversity of extrachromosomal DNA of *A. hydrophila* from aquaculture systems was indicated from plasmid profile, antibiogram, cellular protein profile and RAPD pattern of plasmid DNA.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

INTRODUCTION

Disease outbreaks are recognized as one of the major constraints to aquaculture. Production and trade are adversely affected due to this, resulting in poor economic development and socio-economic implications. Effective control and treatment of the diseases require access to molecular diagnostic tests that are rapid, reliable and highly sensitive. This in turn requires proper understanding of the molecular genetic profile of these pathogens and their genetic diversity.

Supplementation of artificial feeds with antibiotics is practiced by many in the intensive and semi-intensive systems of aquaculture to prevent the spread of diseases and to improve the food conversion ratios. This leads to the development of increased antibiotic resistance in the bacterial pathogens of the aquaculture system. Plasmids have been reported to be the mediators for resistance to a number of drugs in bacterial pathogens. This has led to an interest in the study of plasmid profile and its association with drug resistance.

Of the different species of the genus *Aeromonas* which are autochthonous inhabitants of aquatic environments *Aeromonas hydrophila* is of particular interest. It had received attention in the past because of its association with acute or chronic gastroenteritis in humans following ingestion of contaminated food and water. Bacteria of *Aeromonas hydrophila* group occur widely in aquatic environments and have been implicated in the aetiology of a variety of systemic and localized diseases in fish and reptiles (Son *et al.*,1997)

Plasmids play a number of important roles in the lives of bacteria. Most of the bacterial species are pathogenic because of the virulence factors encoded by their plasmids. Plasmids are small, circular DNA

molecules that can exist independently of host chromosomes and are found in many bacteria. They have their own replication origins and are autonomously replicated and stably inherited. Plasmids have relatively few genes, generally less than 25-30. These genes code for a number of macromolecules like toxins, drug resistance factors, degradative enzymes, etc. which render the bacterium better equipped to establish itself in the host. Single copy plasmids produce only one copy per host cell. Multicopy plasmids may be present at concentrations of 40 or more per cell.

The genes for drug resistance are found on both the bacterial chromosome and plasmids. Spontaneous mutations in bacterial chromosome, although they do not occur very often, will make bacteria drug resistant. Frequently, a bacterial pathogen is drug resistant because it has a plasmid bearing one or more resistance genes, such genes are called 'R' factors. Resistance genes often code for enzymes that destroy or modify drugs. Once bacterial cell possesses plasmids with an 'R' factor, it may be transferred to the other cell quite rapidly through normal gene exchange processes such as conjugation, transduction and transformation. Because a single plasmid may carry genes for resistance to several drugs, a pathogen population can become resistant to several antibiotics simultaneously.

In case of intensive aquaculture, where antibiotics are being extensively used as a prophylactic measure, several fish pathogens have developed resistance. There is the possibility for the transfer of resistance plasmids to human pathogens, which in turn may also develop resistance. Thus, the study and constant monitoring of the plasmid profile of the bacterial population becomes important.

A number of epidemiologically important characters of the bacteria are reported to be plasmid dependant and therefore, characterization of the bacteria in respect of its plasmid profile assumes importance. Microbial molecular genetics is gaining importance in recent times as a reliable way for the classification and identification of microbes. Arbitrary/Random primed

amplification of polymorphic DNA (AP-PCR / RAPD) has been increasingly reported as a method for the genetic characterization of microorganisms. The RAPD profile is highly discriminatory between isolates within species and is an ideal tool for evaluation of bacterial genetic heterogeneity which may not be revealed by other methods. An attempt is made in this work to generate RAPD profile using plasmid DNA as the template.

Proteins are the instruments by which genetic information is expressed. There are thousands of different kinds of these macromolecules in the cell, each one carrying out a specific function. Their structure and function are determined by the gene encoding each one of them. Protein profile has wide applications in the characterization of bacteria. A variety of functions like drug resistance, antigenicity, etc in bacteria were found to be associated with their proteins, many of which in turns are supposed to be under the control of plasmids.

Pathogenesis of most of the bacterium are attributed to the proteins secreted by them. In the study of pathogenic mechanism of the bacterium there has been much interest regarding the role of these substances as toxins, aggressins, etc.

cellular bacterial proteins are specific to each bacterium in their number and molecular weight. *Aeromonas hydrophila* produces a large variety of extracellular products including haemolysins, aerolysins, cytotoxins, enterotoxin and cytolytic enterotoxins.

Hence, the present work was undertaken with the objective of studying the plasmid DNA profile, drug resistance patterns, RAPD profile of plasmid DNA and cellular protein profile of *Aeromonas hydrophila* isolates from aquaculture systems.

2.Review of Literature

REVIEW OF LITERATURE

2.1. Plasmids

Plasmids are self-replicating extrachromosomal DNA molecules found in both Gram negative and Gram positive bacteria as well as in some yeast and other fungi. Although most of them are covalently closed circular double-stranded DNA molecules, linear plasmids have also been recently isolated from bacteria. In general, plasmids are not essential for the survival of bacteria, but they may nevertheless encode a wide variety of genetic determinants, which permit the host bacteria to survive better in an adverse environment or to compete better with other microorganisms occupying the same ecological niche (Luis *et al.*, 1998).

The term plasmid was originally used by Lederberg to describe all extrachromosomal hereditary determinants and it is currently used to describe autonomously replicating extrachromosomal DNA of bacteria. Plasmid size varies from a few to several hundred-kilo bases (Kb) and bacterial cells can harbour more than one plasmid species. Plasmids are found in a variety of microorganisms and it is difficult to generalize about plasmids.

There are a number of features that are not found in all plasmids but which are common enough that they deserve mention below.

1) Many plasmids contain genes uninvolved in either replication or incompatibility. Such genes can encode properties like antibiotic resistance ('R' factors), degradation of complex macromolecules, production of bacteriocins, resistance to various heavy metals, synthesis

of antibiotics or virulence factors necessary for infection of animal or plant hosts. Clearly, virtually any gene can be found on a plasmid.

2) A second common property is the ability to the transfer of the plasmid itself from one cell to another, termed conjugative ability. Conjugation is defined as the unidirectional transfer of genetic information between cells by cell-to-cell contact.

3) Plasmids have mechanism that increases the likelihood that, following cell division, both daughter cells contain a copy of plasmid. This is effected by variety of mechanisms including monomerization of plasmid multimers and association of plasmid with membranes (which apparently helps physical separation of the plasmids).

2.2. Role of plasmids

There are various functions that can be encoded on a plasmid. An obvious way of classifying plasmids is by function. There are five main classes.

1) Fertility (F) plasmids, which contain only tra genes. Their only function is to initiate conjugation.
2) Resistance (R) plasmids, which contain genes that can build a resistance against antibiotics or poisons.
3) Col-plasmids, which contain genes that code for the production of colicines, proteins etc. that can kill other bacteria.
4) Degradative plasmids, which enable the digestion of unusual substances e.g. toluene or salicylic acid.
5) Virulence plasmids, which turn the bacterium into a pathogen.

Role in antibiotic resistance:

Kirby (1978) reported that many characteristics including resistance to antibiotics and other agents in Eubacteria were determined by plasmids.

Toranzo *et al.* (1983) reported that loss of resistance to tetracycline in *A. hydrophila* strains was associated with loss of plasmid content in susceptible derivatives.

R-plasmid mediated drug resistance was demonstrated by Liu *et al.*,(1987) in 37.98% of pond water isolates and 59.70% of diseased eel isolates of *A. hydrophila*.

Kontny *et al.* (1988) analyzed samples of river water for the occurrence of antibiotic resistant *Aeromonas* and found that 21.2% of the 826 *Aeromonas hydrophila* strains tested harboured R-plasmid. Out of 15 strains, 11 strains harboured transferable and 4 strains harboured non-transferable multiple antibiotic resistance plasmid DNA of 100 MDa.

Chowdhury *et al.* (1994) detected transferable R-plasmid for the OT resistance in *Aeromonas spp.* isolated from fresh water aquaculture facilities of Bangladesh.

Barnes *et al.* (1991) examined *Aeromonas salmonicida* for susceptibility to the beta-lactam antibiotic amoxycillin and found that resistance is chromosomal rather than plasmid mediated.

Amita Jain *et al.* (1993) found correlation between plasmid pattern and drug susceptibility pattern in *Shigella dysenteriea*.

Inglis *et al.* (1993) isolated forty oxytetracycline-resistant strains of *Aeromonas salmonicida* and tested them for susceptibility to 12 antibacterial agents. There were 10 resistance patterns with multiple

resistance to 2-6 antibacterial agents. He found that transferable R-plasmids encoding oxytetracycline resistance were present in 11 out of the 40 isolates.

Transferable R-plasmids were detected only from the drug resistant strains of *A. hydrophila* isolated by Saitanu *et al.* (1994) from Thailand. Transferable R-plasmids were reported in *A. hydrophila* isolated from Japan (Aoki *et al.*, 1971), Taiwan (Kou and chung, 1980) and USA (Shotts *et al.*, 1975).

Sadaa (1994) reported that the plasmid pRAS1 in *Aeromonas salmonicida* encodes tetracycline resistance. Noonam and Trust (1995) also reported that A. *salmonicida* possessed plasmids of varying sizes and encoded antibiotic resistance elements.

Sobecky *et al.* (1997) reported that the analysis of 297 plasmid-bearing isolates from aerobic heterotrophic bacteria did not demonstrate a correlation between plasmid content and antibiotic or heavy metal resistance traits.

Aoki (1998) detected R-plasmid encoded resistance to the following antibiotics i.e. chloramphenicol, sulfamonomethonine, kanamycin and tetracycline, in *Aeromonas salmonicida*.

Adams *et al.* (1998) reported that oxytetracycline resistance of *Aeromonas salmonicida* isolates was encoded by high-molecular-weight R-plasmids that were capable of transferring OT resistance to both environmental and clinical isolates of *Aeromonas spp.*

Rhodes *et al.* (2000) reported that the plasmids encoding tetracycline resistance have disseminated between different *Aeromonas spp.* and *E. coli,* and also between the human beings and aquaculture environments in distinct geographical locations.

Schmidt *et al.* (2001) reported that 40 out of 216 resistant Aeromonads harboured large plasmids and found a positive correlation between conjugative R-plasmids and *tetA* among the OTC resistant aeromonads isolated at Danish rainbow trout farms.

Other roles:

Virulence factors of certain bacterial pathogens are encoded by the plasmids. The correlation between enhanced virulence and presence of a 50MDa plasmid in *V. anguillarum* was reported by Crossa *et al.* (1977).

Plasmids play important role in adaptation of *Pseudomonas spp.* to chronic petroleum pollution. Devereux *et al.* (1982) isolated oil degrading bacteria from oil spills on industrial bay and off shore oil field and grew them on liquid enriched media of crude oil and poly nuclear aromatic hydrocarbons. He observed the presence of plasmids in 21% of strains from crude oil and 17 strains from poly nuclear aromatic hydrocarbons. Fifty percent of the strains had multiple plasmids.

Chromosomal replication origin (Oric) was isolated from plasmid of *V. harveyii* by Zyskind *et al.* (1983). The Oric was found to be functional in *E. coli.*

Plasmid encoded mechanism for manganese oxidation by bacteria was studied by Colwell *et al.* (1986). Marine bacteria are able to survive in polluted environment due to the presence of self transmissible plasmids, since they are able to transfer plasmid DNA coding for ecologically advantageous functions such as detoxification of heavy metals, oxidation of manganese etc. Van-waasbergen *et al.* (1993) studied role of plasmids in the oxidation of manganese. Gregory *et al.* (1982) reported that heterotrophic bacteria, oxidizing manganese, lost the capacity along with loss of plasmid when maintained in laboratory.

Bell and Trust (1989) observed that A. salmonicida harbours 17 plasmids in the size range of 12 to 90 KDa, which encodes various proteins.

Plasmids can be used for the transformation of bacteria as cloning vectors (Hackett and Das Sarma , 1989).

Borrego *et al.* (1991) studied the relationship between virulence characters and the presence of plasmids in the isolates of *Aeromonas hydrophila* collected from shellfish and water and found that 60% of the strains simultaneously possessed plasmids and haemolytic activity.

Yersinia spp. harbour plasmid encoding for virulence in all three pathogenic strains namely *Y. pestis*, *Y. pseudotuberculosis and Y. enterocolitica* (Wolf Watz et al., 1991).

Studies on plasmids in waste water bacteria showed that most of them had multiple (2-4) plasmids and have direct correlation between the number of plasmids and speed of crude oil degradation by bacteria (Floodgate, 1991).

Singer *et al.* (1992) observed that virulence plasmid pJM1 of *V. anguillarum* mediates the restriction system that prevents the conjugal transmission of plasmid DNA from *E. coli* donor into *V. anguillarum* (pJM1).

Saitanu *et al.* (1994) found plasmid-encoded resistance to tetracycline in 9 of 54 resistant strains of *Aeromonas hydrophila* isolated from cultured snake head (*Ophicephalus striatus*).

Boettcher *et al.* (1994) reported that plasmids of bacteria *V. fisheri* carry genes that are important for the survival of these strains outside the sepiolid squid symbiont.

Plasmids in *V. parahaemolyticus* controls biodegradation, polymyxine resistance and low level halophilism like characteristics (Chakrabarti *et al.*, 1994).

Shields *et al.* (1995) isolated an aromatic degradative plasmid TOM measuring 70 to 100 Kb from Burkholderia (Pseudomonas) capciaG4.

Yasuda *et al.* (1995) observed that plasmid affect the temperature tolerance in acido-thermophilic archaebacterium, *Thermoplasma.*

Nicholson *et al.* (1995) observed that cystiene synthesis by *Synechococcus spp.* is mediated by plasmids of size 8 and 48.5 Kb.

Naidu (1997) reported that there is little effect of plasmids on the production of siderophore by the *Aeromonas hydrophila* strains obtained from diarrheal samples of human patients and fresh water ponds.

2.3. Plasmid profile

The plasmid profiles of a number of bacterial species have been studied by various workers. Many of the studies cover the different strains within the species, isolated from different locations. Plasmid profiles of different bacteria reported by these workers are presented hereunder.

Toranzo *et al.* (1983) studied plasmid profile of fish pathogens *A.salmonicida, A.hydrophila, Vibrio anguillarum, Pasteurella piscicida, Yersinia ruckeri, Edwardsiella tarda* and *Renibacterium salmoninarum.* Out of total of 38 strains, 28 were found to harbour one or more plasmids, with the majority of strains having multiple plasmids. Five strains of *A. salmonicida* possessed six plasmid bands. Four isolates of *P. piscicida* shared three plasmid bands and five *A. hydrophila* strains harboured a common plasmid having a molecular weight of 20 to 30Kb. The highest-molecular-weight plasmids (145 and 130Kb) were detected in *V. anguillarum.*

Hong (1985) isolated plasmids from halophilic bacteria and molecular size of plasmid in *Vibrio spp.*, *Alkaligens spp.*, *Pseudomonas spp.*, *Flavobacterium spp.*, *Bacillus sp.*, *Alkaligens spp.* were 7.2 Kb, 6.4 Kb, 6.85 Kb, 8.5 Kb, 8.75 kb and 6.8 kb respectively.

Lobb and Rhoades (1987) observed two plasmids of 5.7 kb and 4.9kb in *Edwardsiella ictaluri*, the causative agent of enteric septicemia of channel catfish.

Bast *et al.* (1988) reported that strains of *Aeromonas salmonicida* the agent of furunculosis have fairly uniform plasmid pattern. The pattern consisted of four small plasmids of 4.2, 3.6, 3.5 and 3.3 MDa and a larger plasmid. The larger plasmid was most often 50-56 MDa, but sometimes it was even larger. An additional plasmid was present in a few strains. The work carried out by Toranzo *et al.* (1991) also revealed similar plasmid profile in the *A. salmonicida.* However Bell and Trust (1989) observed 2-9 plasmids in strains of A. salmonicida.

Thermophilic bacteria also have been reported to harbour plasmids. Plasmids of 6 MDa and 47 MDa were isolated from *Thermus thermophilus* (Fee and Mathew, 1988). Hyperthermophilic archaebacterium that grows in temperature range of 68 to 101.5°C harbours a plasmid of 3.45 kb pGTS (Charbonnier *et al.*, 1992). Yasuda (1995) reported a plasmid pTAI of 15.2 kb in isolates of an acidothermophilic archaebacterium, *Thermoplasma acidophilum.*

Zhao and Aoki (1992) reported that *Pasteurella piscida* isolated from *S. quinqueradiata* have one large plasmid of 110Kb and two small plasmids of 3.5 and 5.1 Kb which were shared by all strains. However, Margarinos *et al.* (1992) found common plasmid band of 20 and 7 MDa in all *Pasteurella piscicida* studied. But the European strains were found to have an additional 50 MDa plasmid.

Jain *et al.* (1993) screened the plasmid pattern of 32 isolates of *Shigella dysenteriae1* from different parts of India and reported that all the strains had at least five plasmids of following sizes viz. 120 MDa, 57 Mda, 10.5 Mda, 6.5 MDa and 2.5 MDa. Maximum numbers of plasmids seen were eight while minimum numbers of plasmids present were five.

Studies carried out by the Dalsgaard (1994) and Pederson *et al.* (1996) revealed that *Aeromonas salmonicida* strains have 2 to 3 plasmids and all of them share a common small sized plasmid. The strains of *A. salmonicida* from Atlantic coast was found to posses 4 to 6 plasmids within the size range of 4.3 to 8.1 Kb and while the strains from Pacific coast possessed 6 plasmids in the range of 4.2 to 0.9 Kb.

Pettibone *et al.* (1996) reported that out of 74 strains of *Aeromonas*, 36% (27) had plasmid DNA and most plasmid containing strains had multiple plasmids less than 12 Kilo base pairs (Kbp) in size.

Sobecky *et al.* (1997) observed plasmids only in two hundred ninety seven bacteria out of the more than thousand aerobic hydrophilic bacteria isolated from coastal California marine sediments. While majority of the isolates typically contained one large plasmid of 4o to 100 kb size, some contained multiple small plasmids, three to five in numbers with 5 to 10 kb size.

Vibrio salmonicida isolated from Cod and Atlantic salmon have 61,21,3.4 and 2.8 MDa plasmids and a 61 MDa plasmid was found exclusively in *V. salmonicida* strains of Northern Norway (Amaru *et al.*, 1998).

Plasmid profile of *Aeromonas hydrophila*

Twenty-five *A. hydrophila* strains isolated from fresh water fish and water samples were screened for the presence of plasmids by

Noterdaeme *et al.* (1991). Ten strains were found without plasmids while eleven harboured one plasmid of 20kb and four strains contained two or three plasmids. The 20 kb plasmid was common to all plasmid positive strains.

Borrego *et al.* (1991) studied the plasmid profile of sixty strains *Aeromonas hydrophila* isolated from shell fish and water and found that forty strains harboured one or more plasmids. The plasmid profile most frequently detected (15%) was the association of three small plasmids of 4.2, 3.2 and 2.8 MDa.

Thirty-four *A. hydrophila* strains isolated from various fish species and several geographical locations were examined by Ansary *et al.* (1992) for their plasmid carriage and reported that the plasmid occurrence rate was only 14.7 % with size range from 2.6 to 6 MDa. Multiple carriage of plasmid is more likely with strains having three or four plasmids.

Son *et al.* (1997) reported that seven out of twenty-one strains of *Aeromonas hydrophila* isolated from skin lesions of the common fresh water fish (*Telapia mossambica*) harboured plasmids with sizes ranging from 3 to 63.4 kilo base pair (Kb).

Plasmid profile of other microbes

Potts (1984) reported that Cyanobacterium harboured three plasmids of sizes 0.9, 10.0 and 12.0 kb whereas, Vachhani *et al.* (1992), observed two plasmids in non heterocystous filamentous cyanobacterium *Plectonema boryanum.*

Euglena gracilis was reported to contain a circular extra chromosomal DNA of 10.5 kb that constitutes 1% of its total cellular DNA (Cook *et al.*, 1985). Goff *et al.* (1990) found that 25% of red algae studied by him had two or more plasmids. Red algae *Porphyra pukhra* had two large

plasmids of 6859 and 6427 bp and three smaller plasmids of 1896, 2100 and 2101 bp (Moon, 1997).

Hildebrand *et al.* (1992) and Jacob *et al.* (1992) reported that two small plasmids Pcf1 and Pcf2 of size 4273 bp and 4079 bp respectively were present in the marine diatom *Cylindrotheca fusiformis.*

All strains of *Cytophaga psychrophila* harbour four different plasmid profile, Dalsgaard (1993).

Laclaire *et al.* (1997) found that seven genera of the order Siphonocladales and two genera of cladophorales harbours low molecular weight plasmid of 1.5 to 3 Kb.

Koul *et al.* (1997) reported that *Erwina herbicola* ATCC 21998 have two plasmids pVQ1 and pVQ2 of molecular weights 7.4 and 8.0 Kb.

2.4. Importance of plasmid profile

Plasmid profiling can be used in the characterization and identification of bacteria. Plasmid profiles can be taken as a fingerprint in identifying the bacteria. Reud and Boyle (1980) used the same to identify *Edwardsiella ictaluri* that causes enteric septicemia in channel cat fish.

According to Lobb and Rhoades (1987) restriction profiles of the separated plasmids reveal whether the plasmids are closely related or not .

Plasmid profiling is simple and easy to perform, useful in characterizing strains of pathogenic bacteria like *Salmonella typhimurium*, (Olsvick, 1989).

Wilk (1989) reported that plasmid profile along with serological and biological properties is helpful in isolating *V. anguillarum* stains from diseased fish. He classified *V. salmonicida* using the same technique.

Zhao and Aoki (1992) reported that plasmid profile could be used as a fingerprint of bacteria. They found that a plasmid of 5.1kb size is specific to *Pasteurella piscida*, which causes haemophilus influenza.

Willems *et al.* (1993) detected *Coxiella burnetti* in clinical samples by nested PCR approach with primers based on conserved plasmid sequences.

Plasmid profiling was reported to be one of the methods used in epizootological work especially in the isolation and identification of *A. salmonicida* from salmonids affected with furunculosis (Soerum and Kvello, 1993). Dalsgaard (1994), characterized a typical *Aeromonas hydrophila* using plasmid profile.

Austin and Adams (1995) are of the opinion that plasmid profiling can be used as a rapid diagnostic technique for detection of furunculosis caused by *Aeromonas salmonicida*. However Dahlberg *et al.* (1997) observed that plasmid types isolated from different habitats and from different sampling occasions showed little similarity indicating high variation.

2.5 PCR based molecular characterization of microbes

PCR is a technique for the *invitro* amplification of DNA, which lies between two regions of known sequence. American biochemist Kary B. Mullis conceived the idea of PCR in 1983 and it was later developed into a technique by Mullis and his associates at the Cetus Corporation in

Emeryville, California. This technology has proven to be a revolutionary method, which gives scientist the great advantage of generating a large number of target DNA sequences from trace amounts of DNA material. Since it's introduction was first reported by Saiki *et al.* (1985), PCR has already become a wide spread technique in research laboratories. Random primed / arbitrary primed PCR which generates RAPD profile is one of the many modifications of PCR principle, which can be used for molecular genetic characterization of the microbes.

AP-PCR / RAPD profiling of the genomic DNA of a number of bacteria have been reported. Brousseau *et al.* (1993) conducted arbitrary primed PCR of *Bacillus thuringiensis*. The AP-PCR products have been reported by him to reveal polymorphisms in DNA. A range of *Bacillus thuringiensis* isolates were typed by him using randomly deigned primers.

Goarant *et al.* (1999) conducted the molecular typing study on *Vibrio* strains implicated in shrimp disease outbreaks in New Caledonia and Japan by using AP-PCR. It allowed rapid identification of isolates at the genomo species level and studies of intraspecific population structures of epidemiological interest. Clusters identified within the species *Vibrio penaeicida* were related to their area of origin, allowing discrimination between Japanese and New Caledonian isolates.

Youssef *et al.* (1999) Characterized twenty-nine clinical isolates of *Klebsiella pneumoniae* obtained from 17 children with malnutrition by antibiotic susceptibility, plasmid analysis and random amplified polymorphic DNA (RAPD) techniques. He reported that RAPD analysis using a single (10-mer) demonstrated that the isolates that have the same antibiotype and the same plasmid profile had different RAPD fingerprint patterns showing that the RAPD technique is better than antibiotype characterization and a plasmid analysis profile.

A comparison of AP-PCR, antibiotic resistance and plasmid profile of *Salmonella enteritidis* isolated from fish was conducted by Radu *et al.* (2000). The AP-PCR result showed that the collection of isolates were genetically very heterogenous. Their results also demonstrated that AP-PCR method was more sensitive than plasmid profiling and antibiotic resistance patterns with respect to the individualization of the isolates.

Divya *et al.* (2002) compared the discriminatory efficiency of RAPD profile, protein fingerprinting and biochemical characterization of *Aeromonas hydrophila* and concluded that RAPD of genomic DNA is highly discriminatory.

However, evaluating the plasmid DNA diversity using the random primed PCR amplification of plasmid DNA remains to be attempted in bacterial pathogen like *A. hydrophila*.

2.6. Drug resistance

One of the serious problems faced today in the treatment of infectious disease is the development of bacterial resistance to the antibiotics being used. An organism that has become resistant to a particular chemotherapeutic agent is said to be drug-fast or drug-resistant. The development of resistance may be due to the ability of an organism to destroy the antibiotic or due to a mutation that allow the organism to bypass the sensitive step inhibited by the antibiotic, that causes the cell to become impermeable to the antibiotic. An extremely important and interesting problem in drug resistance was first reported in 1956 in the genus *Shigella*. The unusual nature of drug resistance in *Shigella* was that each resistant organism was resistance to several drugs such as sulfonamide, streptomycin, chloramphenicol and tetracycline (Volk and Wheeler, 1980).

Plasmids often confer antibiotic resistance to the bacteria that contain them. R-factors or plasmids have genes that code for enzymes

capable of destroying or modifying antibiotics. Some R-plasmids have only a single resistance gene, while others can have many. In many cases, the resistance genes are within a transposon and thus it is possible for bacterial strains to develop multiple resistance plasmids. R-plasmids are self-transmissible. Conjugation transfers the plasmid from one bacteria to other causing rapid spread of the drug resistance.

Baya *et al.* (1986) screened the bacterial isolates collected from sewage effluents for the presence of plasmid DNA and susceptibility test against nine antibiotics. He found that isolates from toxic chemical water were more resistant to antimicrobial drugs and more plasmid content than strains of sewage-impacted water or uncontaminated water.

Liu *et al.* (1987) investigated drug resistance in *Aeromonas hydrophila* strains isolated from eel (*Anguilla japonica*) ponds and diseased eels. He found that 79.16% of the isolates from the pond water and 98.53% from diseased eels showed resistance to one or more antimicrobics.

Tsoumas *et al.* (1989) reported that repetitive use of 50% MIC concentrations of 4-quinolone antimicrobials against selective isolates of *Aeromonas salmonicida* colonies resulted in a 7.2X increase in resistance in 12 generations.

The genes for drug resistance are found on both the bacterial chromosomes and plasmids (Prescott *et al.*, 1990). Spontaneous mutations in the bacterial chromosome, although they do not occur very often, will make bacteria drug resistant. Usually, such mutations cause a change in the drug receptor; therefore, the antibiotic can not bind and inhibit.

Dixon *et al.* (1990) determined the antimicrobial resistance among *Aeromonas* isolates from tropical fishes. He reported that *A. sobria* was most resistant, often showing resistance to 9 out of the 12 drugs tested. On the other hand *A. hydrophila* was consistently the least resistant.

Borrego *et al.* (1991) studied the plasmid profile and their association with antimicrobial resistance in *A .hydrophila* and found that out of 60 strains only two were susceptible to all the antimicrobial agents tested. The highest incidences of resistance were to tetracycline (96.7%), prystanamycin (93.3%), ampicillin (91.7%), and cephalothin (91.7%).

Kaznowski *et al.* (1991) characterized 106 isolates of motile *Aeromonas spp.* for their resistance to antimicrobial agents and found that susceptibility among the three species of the motile *Aeromonas* (*A.salmonicida, A.caviae, A.hydrophila*) were similar to all the agents tested except cephalothin. All of the isolates were susceptible to kanamycin, nalidixic acid, tobramycin, amikacin, netilmicin, cefuroxime, ceftriaxone, cefoperazone and cefotaxime.

Barnes *et al.* (1991) examined eight isolates of *Aeromonas salmonicida* collected from outbreaks of furunculosis in farmed and wild Salmon (*Salmo salar*) for susceptibility to the beta-lactam antibiotic amoxycillin. He found that all isolates were resistant to amoxycillin and suggested that resistance is chromosomal rather than plasmid mediated.

Ansary *et al.* (1992) isolated *A. hydrophila* strains from various fish species and several geographical locations in Malaysia and found that all strains possess multiple resistance, most commonly to ampicillin and carbenicillin. Six different antibiogroups representing 18 different antibiotic patterns were obtained and rifampicin was most active antibiotic against these strains.

Aoki (1992) reported that the regular practice of using chemotherapeutic agents against bacterial infections in fish farms in Japan increased the incidence of drug resistant strains of fish pathogenic bacteria .i.e. *Aeromonas hydrophila*, *A. salmonicida, Edwardsiella tarda, Pasteurella piscicida*, non haemolytic *Streptococcus species* and *Vibrio anguillarum.* Fish

pathogens carrying R-plasmid with multiple drug resistance have been widely distributed in fish farms.

Chowdhury *et al.* (1994) reported that the sensitivity patterns of the OT *Aeromonas spp.* were variable to the other antimicrobial agents i.e., oxolinic acid, chloramphenicol, trimethoprim, streptomycin, amoxycillin, furazolidone, nitrofurantoin, kanamycin, nalidixic acid, novobiocin and ampicillin.

Strains of *Aeromonas hydrophila* isolated by Saitanu *et al.* (1994) from cultured fishes, human, water of fish ponds and soft-shell turtles in Thailand, showed intrinsic resistance to ampicillin. Out of 68 strains fourteen strains were susceptible to all drugs used except ampicillin, while the remaining 54 strains were resistant to either chloramphenicol, streptomycin, tetracycline, sulfamonomethoxine, cephazoline, trimethoprim or furazolidone

Liu *et al.* (1995) screened the *Aeromonas hydrophila* and *Edwardsiella tarda* for the presence of transferable R-plasmid and drug resistance. Transferable R-plasmids were present in 5 out of 53 resistant isolates of *A. hydrophila* and in 3 out of 53 resistant isolates of *E.tarda.* He found that 38.30% of the *A. hydrophila* and 25.60% of the *E.tarda* isolates were resistant to one or more of the antimicrobials tested.

Requelme *et al.* (1996) found that bacterial population of dying larvae of *Argopecten purpuratus* was composed of only *Aeromonas hydrophila* strains, which proved tc be resistant to most of the chemotherapeutic agents tested.

Pettibone *et al.* (1996) isolated *Aeromonas* strains from brown bull head (*Ictalurus nebulosus*) and found that all strains had multiple antibiotic resistance with most strains being resistant to rifampicin (97%), novobiocin (96%) and vancommycin (85%). No relationship between plasmid content and antibiotic resistance was found.

Son *et al.* (1997) screened isolates of *A. hydrophila* for the presence of plasmid DNA and sensitivity to antimicrobial agents and reported that all fish isolates were resistant to ampicillin and sensitive to gentamycine. Most isolates were resistant to streptomycin (57%), tetracycline (48%) and erythromycin (43%).

Singh *et al.* (1997) found a common multiple drug resistance among the enterotoxigenic strains of *Aeromonas hydrophila* isolated from fresh water fishes.

Bacterial resistance to antimicrobial drugs has become wide spread in some areas of aquaculture. Cultured fish have been reported to be infected with resistant strains of *Aeromonas hydrophila, A. salmonicida, Edwardsiella ictaluri, Pasteurella piscicida, Vibrio anguillarum, Y.ersinia ruckeri, Streptococci* and others (Dixon, 2001).

2.7. Cellular protein profile

Proteins are the expressions of the genetic information coded in DNA. Therefore, the protein profile comparison shall reveal the functional genetic diversity. The protein profiles of some of the bacterial pathogens reported by different workers are presented below.

On the basis of structural protein analysis along with morphology and DNA homology, Conveney *et al.* (1987) characterized and compared four lactic streptococcal bacteriophages.

Hellwig *et al.* (1988) compared the outer membrane proteins and surface characteristics of four adherent and one non-adherent mutant of *Bordetella avium* and showed that adherent phenotypes had identical protein profiles while non-adherent organisms lacked five of the proteins present on adherent organisms.

Nine strains of *Renibacterium salmoninarum* isolated from different geographical locations were compared by Daly and Stevenson (1990) for protein profiling by SDS-PAGE. Profile of seven of these strains as well as the type strain ATCC-33207 were similar in having a major protein of 57 KDa and a minor protein of 58 KDa. While one of the remaining strains (Char strain) did not contain the 58 KDa protein, the other strain (MT-239) lacked the both the proteins.

Protein profiles do have a bearing on the drug resistance, antigenicity and genomic function, on many occasions. Bandin *et al.* (1992) had done the analysis of membrane protein and their antigenic properties in a group of 14 geographically diverse strains of *R. salmoninarum*. He detected antigenic heterogeneity with two groups distinctly recognizable.

Cellular protein profile has application in characterization, classification and identification of bacterial isolates, (Mc Lean *et al.*,1993).

Niemi *et al.* (1993) identified the faecal streptococcus from the environmental samples by using protein profile resolved by the SDS gel electrophoresis.

Characterization of six mosquitocidal *Bacillus thuringiensis* strains by Ragni *et al.* (1996) on the basis of protein profiling showed the occurrence of same protein profile and mosquitocidal activity in five strains, the sixth strain showed a different protein profile as well as a noval mosquitocidal activity. Ochiai *et al.* (1997) compared the protein profile of three members of genus *Brachyspira* viz. *B. hydosenteriae, B. innocens and B. pilosicoli* with that of *B. aalborgi* using SDS-PAGE. The profile of *B. aalborgi* was different from others, except for the two heavy protein bands of 49.4 and 52.3 KDa in *B. innocens*.

The protein profiling of Archaea, *Halobacterium halobium*, using two dimensional SDS-PAGE of the whole cell extract revealed the existence of 242 different bands by Nakayama and Masashi (1997).

Gatti *et al.* (1997) studied the profile of cell wall proteins of different species of thermophillic Lactobacilli and found that a protein of approximately 50KDa was characteristic for all of the strains of *L. helveticus* and two protein of about 20 and 30KDa were typical to *L .debrueckoi.*

3. Materials and Methods

MATERIALS AND METHODS

Aeromonas hydrophila isolated from diseased fish as well as water and sediments of the coastal aquaculture systems were used in this study. Isolation was carried out in the Microbiology laboratory of CMFRI. They were tested for the confirmity with the accepted definition.

3.1. Isolation of *Aeromonas hydrophila*

Aeromonas hydrophila were isolated following the standard aseptic procedures, using selective medium, Rimler Shotts agar (HIMEDIA).

Approximately 1gram sample (intestine and gills) from fish were aseptically transferred to a sterilized glass mortar, ground well with pestle and mixed well with 99 ml of sterile distilled water taken in a conical flask. In the case of sediment, 1 g of sample was transferred to a conical flask and shaken well. Water samples were membrane filtered and the filter paper was put in sterile 99 ml distilled water dilutions and shaken in bacteriological shaker for 30 minutes. After thorough shaking, serial dilutions were made according to the standard procedures.

From the sample thus prepared, 1ml was transferred to 10cm sterile glass petridishes and pour plated using Rimler Shotts agar (Shotts and Rimler, 1973). Then the plates were incubated at room temperature (37°C) for 24-48 hours. Yellow colonies without black centers facilitate the rapid identification of *Aeromonas hydrophila* in Rimler Shotts media.

Selected colonies were subcultured in nutrient agar slants and peptone water as stock culture. Subculture was also done at different stages of the study, to carry out different biochemical tests. If the streak plate has more than one morphological type of colony, each type was subjected to the

confirmation steps. The identity of the isolates was confirmed with morphological characteristics and biochemical tests.

3.1.1. Morphological characterization

Morphological characterization including motility test and gram staining reactions were carried out.

1. Hanging drop method

A drop of young culture was transferred to the center of the coverslip. A cavity slide was placed over the coverslip and then inverted so that the drop of culture will be hanging in the center of the cavity slide. Microscopic examination was carried out to find the motility. *A. hydrophila* was found to be motile.

2. Gram's stain

A thin smear of the culture was prepared, air-dried and then heat fixed. The primary staining was with crystal violet followed by the addition of mordant iodine. The crystal violet-iodine complex formed is decolourized with 95% alcohol. Saffranin was added as counterstain to the smear. The bacteria is classified into Gram positive, Gram negative, Gram variable and Gram unreactive forms. *A. hydrophila* is a gram-negative.

3.1.2. Biochemical tests

Pure cultures of *Aeromonas hydrophila* were maintained in nutrient agar slants and peptone broth at room temperature for carrying out the following differential biochemical tests.

1. Oxidase test

For conducting the oxidase test, freshly prepared N', N', N', - Tetramethyl paraphenylene diamine dihydrochloride was poured on a filter

paper strip and the culture is streaked on it using a sterile glass rod. Immediate appearance of a deep purple colour indicated the presence of oxidase enzyme. *A.hydrophila* is oxidase positive.

2. Trehalose fermentation

Oxidase positive colonies were then tested for trehalose fermentation. Trehalose fermentation is determined by inoculating a tube containing 3-10 ml of 0.5% trehalose in purple broth base with a colony from nutrient agar and incubating at 35°C for 24±2 hours. A change in colour of the medium from purple to yellow is considered positive for trehalose fermentation test, indicating the presence of *A.hydrophila*.

3. Catalase test

Hydrogen peroxide was taken in a test tube and a loop containing bacterial culture was dipped into it. The catalase positive reaction is shown by dense bubble of oxygen coming out of the loop, where as negative reaction fails to form the oxygen release. *A.hydrophila* is catalase positive.

4. IMVIC tests

a) **Indole test:** Trypton broth media was inoculated with one loopful of 24-hour nutrient broth culture and incubated for 2-4 days. Into the tube was added 5cc of covac's reagent. The appearance of deep cherry red ring in the reagent layer indicated the presence of indole. *A. hydrophila* is indole positive.

b) **Methyl red- Voges Proskauer test:** MR-VP broth was inoculated with the bacteria and incubated for 3-4 days, at room temperature and then the MR-VP test were carried out

MR test: 5 drops of 0.04 % solution of methyl red was added to the cultured MR-VP broth, mixed well and read at once. A red colour is positive while yellow colour signifies a negative test.

VP test: 0.6 ml of 5% solution of α-naphtha in ethanol and 0.2 ml of 40% KOH are added to 1ml of MR-VP cultured broth. Pink colour in 2-5 minutes and deepening to magenta or crimson in 30 minutes indicates VP positive and negative if it remains colourless.

C) Citrate utilization test: The citrate utilization was found out by inoculating the cultures on slants made from Simmons's Citrate Agar. After incubation period at room temperature, the positive reaction is shown by the development of blue colour in the medium indicating positive result for *A.hydrophila*.

5. Nitrate reduction test

The ability to reduce nitrate was tested in ordinary peptone broth containing 0.3% potassium nitrate inoculated with the culture. Turbidity was checked after the incubation period and the nitrate reduction was tested with Nitrate reagent (α-Methylamine-Reagent A and Sulphanilic acid-Reagent B mixed in equal proportion while doing the test). *A. hydrophila* gives positive reaction for this test.

6.Production of Ammonia or Ammonium salts

Peptone broth was prepared, sterilized and inoculated with a loopful of culture .Tubes were incubated for 3-4 days after which two drops of Nessler's regent was added to the tube. The presence of ammonia in the medium reacting with Nessler's reagent is shown by quick development of

yellow ,orange or brown colour. The intensity of colour indicate the concentration of dissolved ammonia. *A.hydrophila* gives positive result with ammonia production.

3.2 *Aeromonas hydrophila* isolates used for the screening of plasmid DNA

A total of twenty two bacterial isolates, which were identified as *A. hydrophila* on the basis of the morphological characteristics and biological tests were used for the screening of plasmid DNA, antimicrobial sensitivity tests and cellular protein profile. Out of 22 isolates, 12 isolates were from fish, 6 from water and 4 from sediments. These isolates were designated serially from Ah_1 to Ah_{22}.

Nutrient broth cultures of the isolates were made to facilitate the isolation of plasmid DNA and proteins from them. Nutrient broth used for the culture contained 1.5% salt. Incubation was done at 37^0C for 24 hours.

3.3. Isolation of plasmid DNA

3.3.1. Principle

The commonly used protocols for the bacterial plasmid isolation consist of lysis of bacterial cell wall using lysozyme and denaturation of protein and DNA by using alkaline lysis which consist of 1% detergent SDS and 0.2M NaOH followed by adding potassium acetate which neutralizes the media and precipitate chromosomal DNA and most of the protein, leaving plasmid DNA and RNA in the solution. Then phenol/ chloroform-isoamyl alcohol extraction removes any remaining protein in the solution. Plasmid is then precipitated by addition of ice cold sodium acetate and ethanol. This procedure effectively remove the contaminating proteins.

3.3.2. Reagents required

All the chemicals and enzymes used were of molecular biology grade.

1) TEG buffer (pH:8)
   25 mM Tris-Hcl
   10 mM EDTA
   150 mM Glucose
2) Lysozyme (SIGMA, USA)
3) Alkaline lysis buffer
   1% SDS
   0.2N NaOH (Prepared from a stock of 10% SDS and 2N NaOH)
4) 3M Potassium acetate (pH:5.2)
5) Neutral phenol
6) Chloroform – Isoamyl alcohol (24:1)
7) Sodium acetate (pH:5.2)
8) Absolute ethanol
9) 70% ethanol
10) TE buffer (pH:8)

3.3.3. Procedure

The procedure adopted in the present study was a modification of the procedure developed by Maniatis *et al.* (1989).

- Bacteria was inoculated in10ml nutrient broth culture media and incubated for overnight.
- The bacteria were harvested from the broth culture during the post logarithmic phase by spinning at 10,000 rpm for 10 minutes in 1.5 ml eppendorf tube in a refrigerated high speed centrifuge.
- The pellet were washed in 1 ml TEG buffer by spinning at 8000 rpm for 5 minutes.
- The supernatant was carefully drained out and the pellet was suspended in 100 µl of TEG buffer (pH:8) containing lysozyme (5mg /ml). The cell

suspension was vortexed in a vortex mixture and incubated at 4^0C for 10 minutes.

- After the bacterial cell wall lysis in TEG buffer containing lysozyme, 200 µl of alkaline lysis buffer containing 0.2 M NaOH and 1% SDS was added. The solution was mixed gently and incubated at 4^0C for 15 minutes. The solution was gently shaken to mix the contents at every five minutes interval.
- The nuclear DNA and proteins got denatured during alkaline lysis and the solution became viscous. To that viscous solution,150 µl of 3M potassium acetate (pH:5.2) was added and kept at 4^0C for 15 minutes. The contents were mixed well at every five minutes interval. A network of precipitated proteins and nuclear DNA was formed.
- After 15 minutes the preparation was centrifuged for 15 minutes at 10,000 rpm and 4^0C, after centrifugation, the clear supernatant containing plasmid DNA was collected in another micro centrifuge tube.
- To that, equal volume of neutral phenol was added to precipitate any proteins present in the solution. The solution was mixed by gentle shaking and kept undisturbed for 10 minutes to precipitate the proteins.
- The preparation was then centrifuged at 10,000 rpm and 4^0C for 10 minutes. Protein layer got precipitated in the aqueous-organic interphase. The aqueous phase was carefully pipetted out and transferred to another eppendorf tube and the neutral phenol extraction was repeated again.
- To the aqueous phase collected, equal volume of chloroform-isoamyl alcohol was added to remove traces of phenol and other impurities, if any. The mixture was shaken well and centrifuged at 10,000 rpm and 4^0C for 15 minutes. The aqueous phase was carefully transferred to another eppendorf tube.
- Measured the volume of aqueous phase and $1/10^{th}$ volume of 3M sodium acetate was added and mixed well. To the above mixture, 2-2.5 volume of absolute ethanol was added. The mixture was shaken well and kept at -20^0C overnight for precipitating plasmid DNA.

- Next day the preparation was centrifuged at 10,000 rpm and 4^0C for 15 minutes. The supernatant was discarded carefully and precipitate was washed with 70% ethanol to dissolve the salt (sodium acetate). Then the solution was centrifuged at 10,000 rpm, 4^oC for 10 minutes. The supernatant was discarded completely. The pellet was air dried. When the pellet was completely free of moisture, it was dissolved in minimum quantity of TE buffer (pH:8) The plasmid DNA thus obtained was stored at -20^0C, till further use.

3.4. DNA Electrophoresis and visualization of plasmid DNA

Plasmids isolated from the bacteria were subjected to agarose gel electrophoresis to resolve the plasmids according to their size.

3.4.1. Reagents required

1) Agarose (GENEI)
2) 1 x TEB (pH:8.0)
   0.89 M Tris-HCl
   0.02 M EDTA
   0.89 M Boric acid
3) Loading buffer
   Glycerol – 2 ml
   Bromophenol blue (0.5%) –1 ml
   1 x TEB – 7 ml
4) standard DNA marker (λ DNA double digest)
5) Ethidium bromide (1 μg/ml)

3.4.2. Procedure

Preliminary trials of plasmid DNA electrophoresis with different percentages of agarose gel were carried out and 0.8% agarose gel was found to be most suitable for the resolution of plasmid DNA. Therefore, 0.8%

agarose gel prepared in 1xTEB was used for routine screening of plasmid DNA and into that 5μl of the sample was loaded along with a standard DNA marker (λ DNA cut with Hind III).The duration of electrophoresis was three to four hours.

The gel was then stained with ethidium bromide in darkness for 30 minutes. The stained gel was dipped in distilled water to remove excess stain. Then gel was viewed and documented by using a gel documentation system. Plasmid DNA appeared as reddish orange bands and the molecular weight of the plasmid was determined by comparing it with standard.

3.5. Random Amplified Polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR)

Random primed PCR amplification of the plasmid DNA was carried out using Operon decamer random primers. Initially, standardization of the optimum concentration of different components for PCR was done by varying their concentrations. Different concentration of $Mgcl_2$ ranging from 1mM to 2mM in assay buffer was attempted and the one with 1.5mM concentration was found to give better results. Similarly varying pH from 8.5 to 9.5 was attempted and a pH 9 was found to give better amplification. Likewise the concentration of Taq polymerase and dNTP were also standardized as 1.25μM and 200μM respectively. The concentration of plasmid DNA template and primer were also optimized as 50ng and 7.5 pico moles respectively for each PCR reaction. Master mix for PCR was prepared under aseptic condition inside a laminar airflow environment. PCR assay was carried out in a final volume of 25μl. Based on the amplification efficiency, the four primers were short listed for regular screening of the samples. One isolates from each of the plasmid profile category were used for this study.

3.5.1. PCR cycles

Thermal cycling was performed with Perkin-Elmer thermocycler (GeneAmp.PCR System2400). Each of the 45 PCR cycles standardized for this work consisted of denaturation of DNA at 92° C for 1 minutes, primer annealing at 37°C for 1 minutes and primer extension at 72°c for 1 minutes. All PCR samples were subjected to an initial denaturation step at 94°C for 2 minutes and a final extension at 72°C for 10 minutes. PCR products were stored at -20°C until electrophoresis was performed. The amplified plasmid DNA products were resolved through agarose gel electrophoresis.

3.6. Antibiotic sensitivity test

There are a number of standard methods for testing the susceptibility to antibiotics. In present study disc diffusion method was used.

3.6.1. Procedure

The disc diffusion method involves the following steps.

- Media preparation- Disc diffusion assay was performed using Muller-Hinton agar .The pH of agar was between 7.2 to 7.4 and media prepared with distilled water.
- Preparation of plates- Melted agar medium was cooled. The cooled agar medium was poured to a depth of 4 mm in sterile petriplates of horizontal and leveled surface.
- Moisture level of media- Immediately prior to inoculation media was moist and agar surface and petri dish lid was free of droplets.
- Inoculum- Pure culture was used as inoculum. Three to four similar colonies were selected and transferred into about 5 ml of nutrient broth and incubated at 35^0 C for 8-12 hours till light to moderate turbidity developed.
- Inoculation of test media- A sterile cotton swab was dipped into the properly prepared inoculum and rotated firmly against the upper inside wall of the tube to express excess fluid. The entire surface of the Muller-

Hinton plates were inoculated by rubbing with the swab containing the inoculum. The entire surface of the plate was streaked with the swab three times turning the plate 60^0 between each streaking.

- Diffusion discs-The antibiotic discs supplied by a reputated manufacturer were used. A maximum 6 discs were applied to a 9 cm petriplates. The discs were deposited with at least 24 mm apart manually using sterile forceps and pressed gently to each discs to ensure the complete contact between the disc and agar surface.
- Incubation- All plates were incubated aerobically at 37^0 C in an inverted position, in stacks of less than five plates and examined after 24-28 hours.
- Reading of plates- After incubation only zones showing complete inhibition (the point at which no growth is visible) were measured and diameter of the zones were recorded in millimeter.

Table 1. Antibiotics tested

Sl. No.	Antibiotics	Symbol	Conc.
1	Amikacin	Ak	30mcg
2	Amoxycillin	Am	10 mcg
3	Ampicillin	A	10 mcg
4	Bacitracin	B	10μ
5	Carbenicillin	Cb	100 mcg
6	Cefaclor	Kf	30 mcg
7	Cephalexin	Cp	30 mcg
8	Chloramphenicol	C	30 mcg
9	Doxycillin	Do	30 mcg
10	Erythromycin	E	15 mcg
11	Gentamicin	G	10 mcg
12	Lincomycin	L	2 mcg
13	Neomycin	N	30 mcg
14	Nitrofurazone	Nr	10 mcg
15	Norfloxacin	Nx	10 mcg
16	Ofloxacin	Of	5 mcg
17	Oxytetracycline	O	30 mcg
18	Pefloxacin	Pef	10 mcg
19	Penicillin-g	P	10U
20	Vancomycin	Va	30 mcg

The results were interpreted by using the zone size interpretative chart (Bauer *et al.* 1996, Performance standards for antimicrobial disk susceptibility tests, 1993). The antibiogram of all strains were prepared for correlating the antibiotic sensitivity with plasmid profile.

3.6.2. Antibiotics tested

Twenty-two antibiotics were selected to check the resistivity pattern of *Aeromonas hydrophila* isolates. The name, symbol and concentrations of all 20 antibiotics are presented in the Table 1.

3.7. Determination of cellular protein profile

Cellular protein profile of the cellular proteins were developed with both NATIVE and SDS polyacrylamide gel electrophoresis.

3.7.1. Isolation of cellular protein

Aeromonas hydrophila were harvested from 2 ml broth culture during the post logarithmic phase by spinning at 10,000 rpm, at 4°C, for 15 minute in 1.5ml eppendorf tubes in a refrigerated high speed centrifuge. The supernatant was drained off and 100 µl of TEG buffer (pH8) containing 5mg/ml lysozyme was added to each of the pellets and vortexed in a vortex mixer. This was incubated at 4°C for 15 minutes, mixing gently after every five minutes. The cell suspension was then centrifuged at 10,000 rpm, at 4°C, for 15 minutes and the supernatant was collected in eppendorf tubes and stored at -20°C for further use.

3.7.2. NATIVE-PAGE

The electrophoretic principle involve the migration of charged particles under an applied electric field. Polyacrylamide gels are formed by polymerizing acrylamide with a linking agent in the presence of a catalyst (per sulphate ion) and chain initiator (TEMED, NNN'N' tetramethyl ethylene

diamine). The porosity of the gel is determined by the relative proportion of acrylamide monomer to bisacrylamide. Gels are usually referred to in terms of the total percentage of acrylamide and bisacrylamide and most separations are formed using gels in the range of 7-15 %. A low percentage gel (with large pore size) is used to separate high molecular weight protein vice-versa.

Reagents required

1) Acrylamide-bisacrylamide Mix (30%)
   29% acrylamide
   0.9% bisacrylamide
2) Separating gel buffer
   1.8 M Tris-HCl (pH:8.9)
   TEMED is incorporated @ 250 µl/100 ml
3) Stacking gel buffer
   0.5 M Tris-HCl (6.8)
4) Electrode tank buffer
   6.19 M Tris-Glycine (pH:8.3)
5) Ammonium per sulphate (APS) (10%)
6) Sample buffer
   Glycerol
   0.5% Bromophenol blue
   0.5 M Tris-HCl (6.8)
7) Coomassive brillient blue R-250 stain (0.15%)
8) Destaining solution
   15% Methanol
   7% Acetic acid

Composition of separating gel (7.5% PAGE)

Separating gel was prepared for 50 ml by mixing following reagents.

12.5 ml Acrylamide-bisacrylamide
6.25 ml of Separating gel buffer
6.25 ml double distilled water

400 μl of 10% APS in 25 ml of double distilled water

Composition of stacking gel (3.5% PAGE)

Stacking gel was prepared by mixing the following reagents in appropriate quantity.

3 ml Acrylamide-bisacrylamide

6.25 ml Stacking gel buffer

15.8 ml of double distilled water

TEMED – 25 μl

100 μl of APS (10%)

Procedure

Preliminary trials were made to know the standard percentage of separating gel with 7%, 7.5%, 8% and 7.5% separating gel was most suitable for the resolution of protein profile. Gel cassette was set with clamps after proper cleaning and three sides were sealed with Vaseline. The separating gel was poured into the cassette below the well. To correct the meniscus few drops of butanol was poured above the separating gel. Gel was allowed to polymerize for 15-30 minutes. Then butanol was poured off and washed with distilled water then stacking gel was poured above the polymerized separating gel. A gel comb was placed in the stacking gel making sure that there is no air bubble trapped in it and allowed for the polymerization for 15-30 minutes. The polymerized gel was kept for 10 minutes into the refrigerator.

The comb was removed from solidified gel without disturbing the shape of the wells. The polymerized gel was removed from the cassette and transferred over the electrophoretic unit which was cool and circuit was completed. Loading sample were prepared by mixing the sample and sample buffer in the volume of 50 μl and 50 μl respectively and 70 μl of this mix was loaded in the wells and duration of electrophoresis was 3-4 hours. Then the gel was kept in the fixative for one hour, after this gel was stained in

Coomassive brillient blue R-250 stain till the development of bands (1-1.5 hours).Then gel was kept in destainer for overnight. After proper distaining bands were viewed on a transilluminator and recorded.

3.7.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS is an anionic detergent, which binds strongly to, and denatures proteins. The protein-SDS complex carries net negative charges, hence move towards the anode and the separation is based on the size of the protein.

Reagents required

The composition of the chemicals and reagents required for casting of separating gel, stacking gel and running buffer were same as of NATIVE-PAGE except the addition of SDS.

Sample buffer composition

Glycerol
SDS(10%)
0.5% Bromophenol blue
0.5 M Tris-HCl (6.8)
β-mercapto-ethanol

Procedure:

The method in the present study was on the lines of Laemmli *et al.* (1970). The percentage of separating gel was a critical parameter in all electrophoretic separations using discontinuous system of buffer along with stacking gel. Separating gel of 12.5%, 11.5%, 11% were tried, and the ideal percentage was found to be 11.5% concentration. This was selected for the present study. It was prepared from 30% stock of Acrylamide and

Bisacrylamide monomers along with 6% of stacking gel. The concentration of protein sample to be loaded was also standardized for ideal resolution.

Gel cassette was prepared as in the case of NATIVE-PAGE. After polymerization, the comb was removed from the solidified gel without disturbing the shape of the well. Then the gel was placed on the electrophoretic apparatus with electrode buffer ensuring that no air bubbles were entrapped at the bottom of the gel. The sample prepared in the following way was loaded into the wells of the stacking gel and layered with running buffer in order to avoid disturbance to the sample.

Sample preparation:

To 85µl of each of the samples, 65µl of sample buffer is added. Simultaneously 10µl of SDS protein molecular weight marker from Banglore GENEI was mixed with 60µl of sample buffer. The samples were then boiled strictly for 5 minutes and the marker for 1 minute. After loading the sample into wells of the gel, the electrodes were connected to the power pack and a constant voltage of 60 volts was applied until the dye front crosses the stacking gel and it was later increased to 140 volts and the electrophoresis was carried out till the dye front reaches the bottom of gel.

The gel, after completion of electrophoresis was washed gently with tap water to remove excess SDS and fixed in fixative for 2 hours. Then it was stained for Coomassive brilliant blue R-250 for a period of 2 hours and the protein bands were visualized over a transilluminator.

The molecular weights of standard SDS-PAGE molecular marker used were 97.4KDa, 66KDa, 43KDa, 29KDa, 20.1KDa and 14.3KDa. RF value of the standard marker was calculated. A semi-log graph was drawn using the R_f values. The R_f values of unknown samples were calculated and extrapolated using the standard graph to determine the molecular weight.

4. Results

RESULTS

Twenty two bacterial isolates from the aquaculture systems were confirmed to be *Aeromonas hydrophila* following the standard procedures. They grew into yellow colonies without black centers, in Rimler-Shotts's media. *Aeromonas hydrophila* confirmed on the basis of morphological and physiological test were gram negative, rod shaped, motile, oxidase positive and fermentative. These twenty two isolates designated serially from Ah_1 to Ah_{22} were used for further study.

4.1.Plasmid profile

Bacteria were screened for the presence of plasmid DNA following the procedure developed by Maniatis et al.,(1989) with some modifications. Plasmid DNA isolated from all the twenty two *Aeromonas hydrophila* isolates were resolved through agarose gel electrophoresis and were visualized on a UV-transilluminator after staining with ethidium bromide. The molecular size of plasmids were determined by comparison of the relative mobility of plasmid DNA vis a vis that of standard molecular weight DNA markers electrophorised along with it. The plasmid DNA profile of these species resolved in the gel are shown in figures 1 to 3.

All the twenty two *A. hydrophila* isolates were found to contain plasmids. The plasmid profiles of the isolates showed diversity, the variations were in plasmid number and molecular size. The molecular size of plasmid DNA detected in the *A. hydrophila* isolates ranged from 1 to 21 kb.

The plasmid occurrence rate was 100%. The number of plasmids carried by the isolates varied from 1 to 4. However, eight different plasmid profiles were present on account of variations in the molecular size of the plasmid. A plasmid of 21 kb was found to be shared by all isolates.

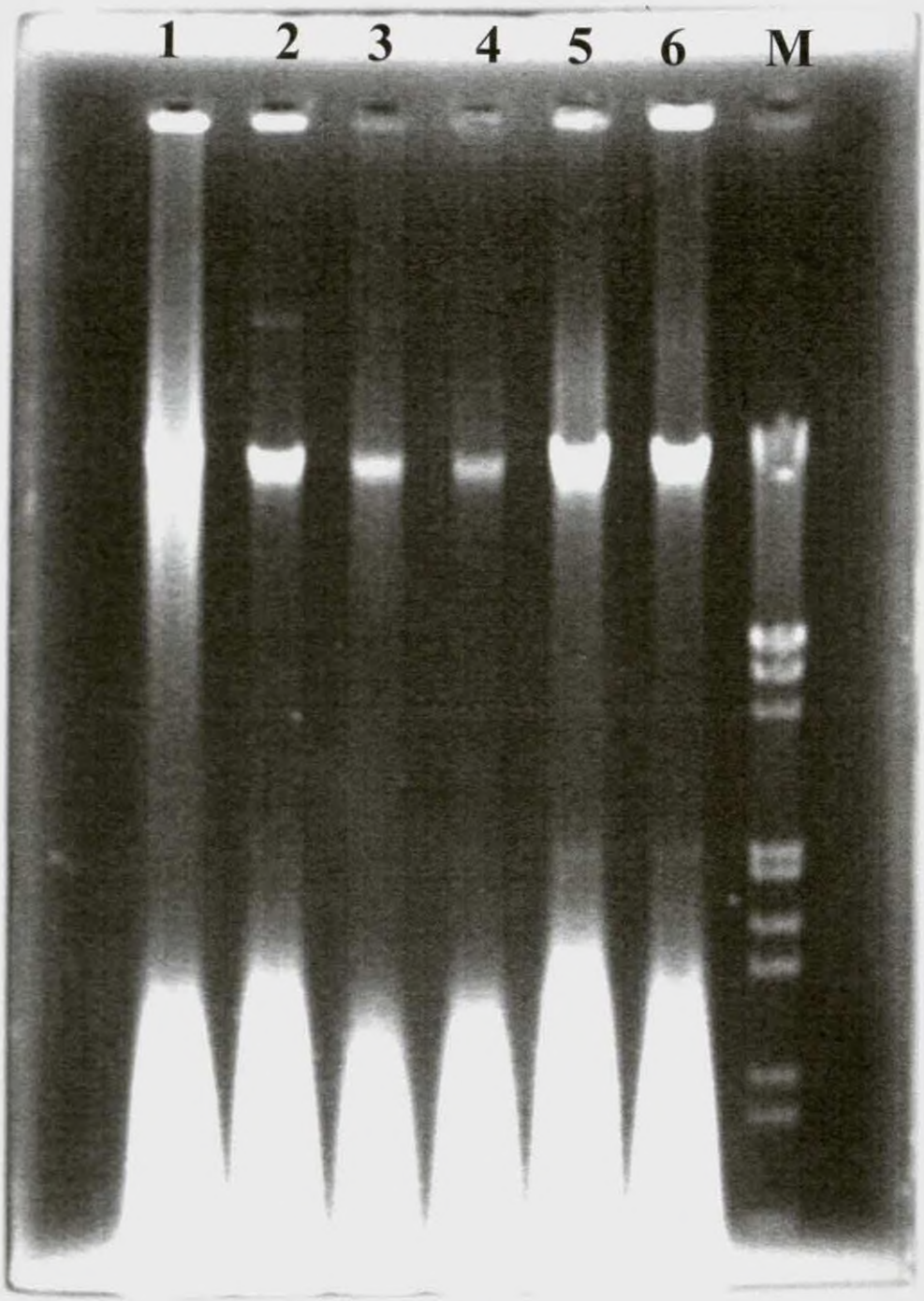


Fig :1

Plasmid DNA profile of *Aeromonas hydrophila* isolates resolved through agarose gel electrophoresis

Lane 1- 6 : Ah_{17}, Ah_{18}, Ah_{19}, Ah_{20}, Ah_{21}, Ah_{22}

Lane M : Standard DNA marker

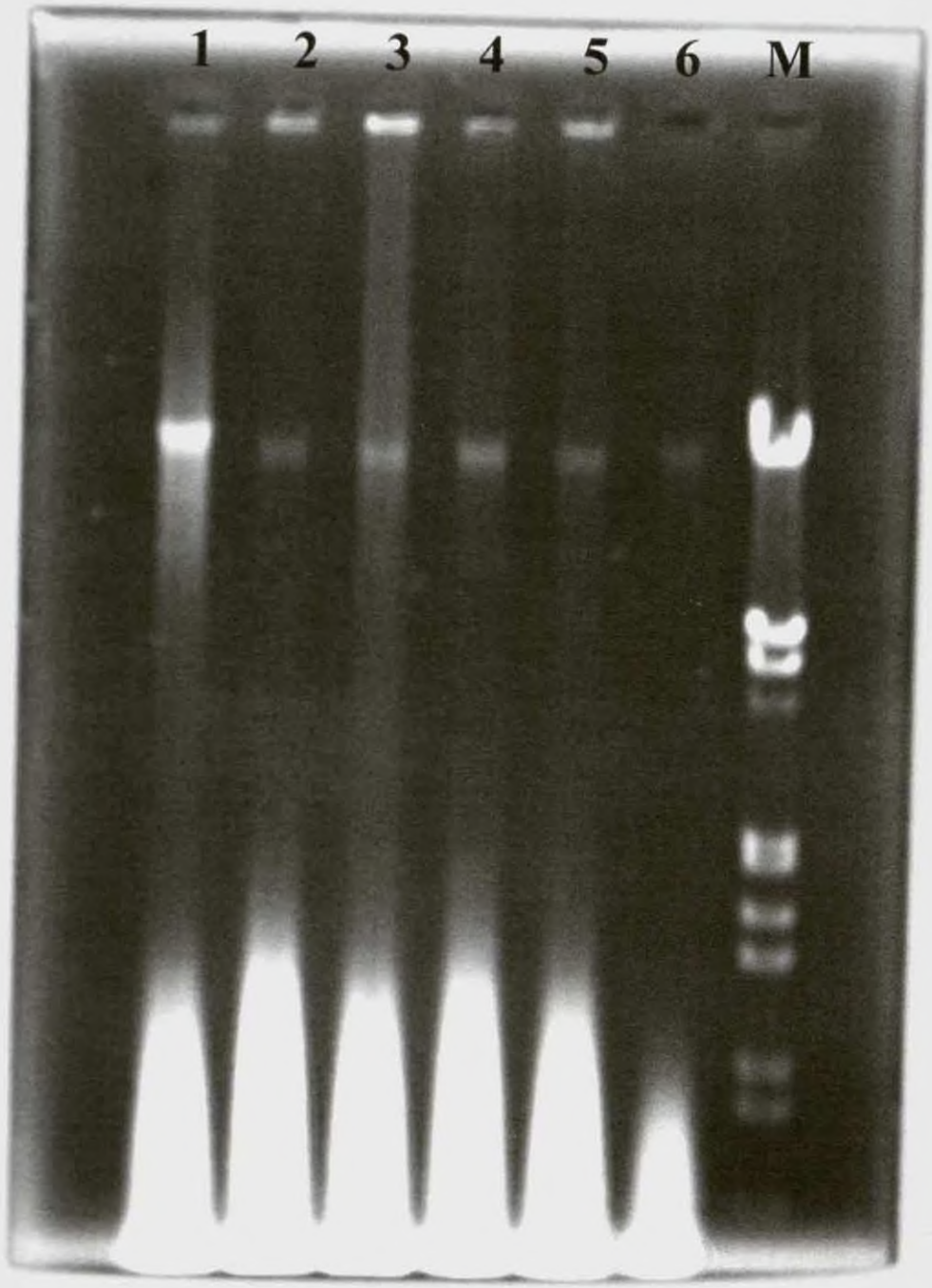


Fig :2

Plasmid DNA profile of *Aeromonas hydrophila* isolates resolved through agarose gel electrophoresis

Lane 1 – 6: Ah_6 , Ah_9 , Ah_{10} , Ah_{11} , Ah_{12} , Ah_{14}

Lane M : Standard DNA marker

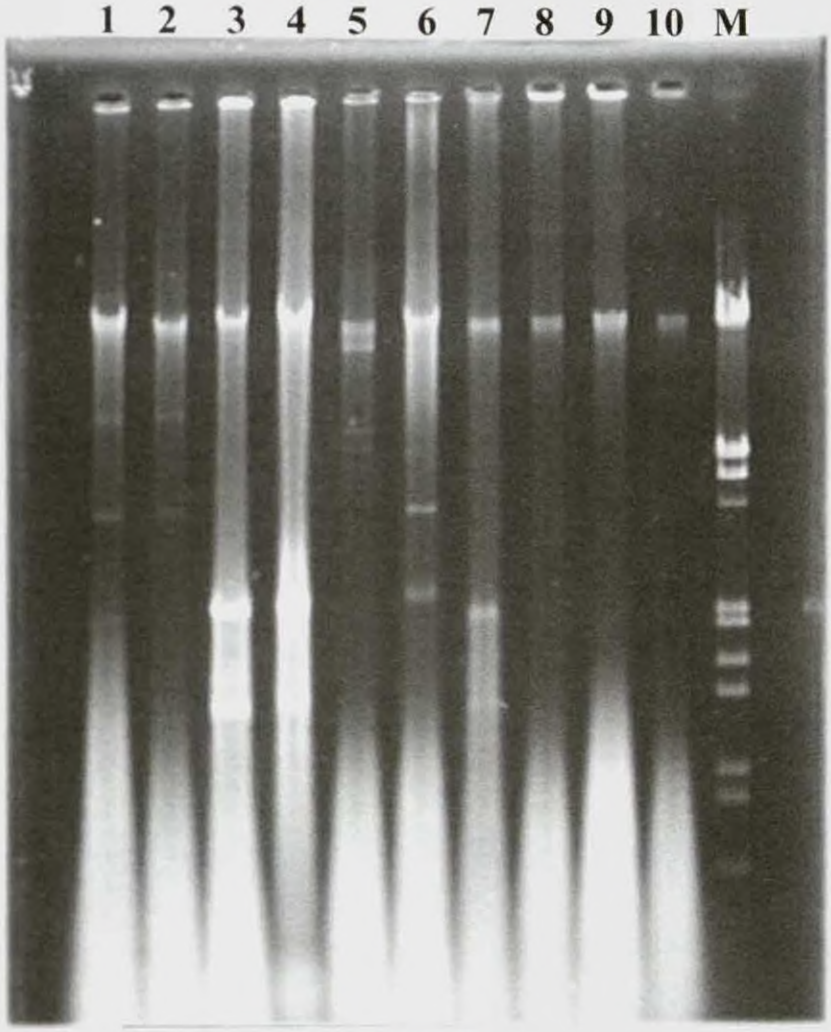


Fig :3

Plasmid DNA profile of *Aeromonas hydrophila* isolates resolved through agarose gel electrophoresis

Lane 1 - 10 : Ah_1, Ah_2, Ah_{13}, Ah_{15}, Ah_3, Ah_5, Ah_{16}, Ah_4, Ah_7, Ah_8.

Lane M : Standard DNA marker

As can be seen from the figures 1 to 3, eleven isolates contained only single plasmid. They were Ah_6, Ah_7, Ah_8, Ah_9, Ah_{10}, Ah_{11}, Ah_{12}, Ah_{14}, Ah_{17}, Ah_{19} and Ah_{20}. All of them carried a plasmid of 21 Kb only and so belonged to a single profile.

Four isolates viz. Ah_4, Ah_{18}, Ah_{21} and Ah_{22} carried two plasmids each. However, two distinct plasmid profiles were evident in them. Isolates Ah_{21} and Ah_{22} harboured a plasmid of 2 kb in addition to 21 kb, the second plasmid in the isolates Ah_4 and Ah_{18} was a mega plasmid whose molecular size was beyond 20 Kb. The exact size could not be determined with the help of marker.

Five isolates (Ah_2, Ah_5, Ah_{13}, Ah_{15}, Ah_{16}) out of 22 isolates harboured 3 plasmids each. However three different profiles in respect of their molecular size were evident. While all of them carried the 21 Kb plasmid, the additional plasmids were of 6 kb and 3 kb size in Ah_2, 2 kb and1 kb in Ah_{13}, Ah_{15} and Ah_{16}, and 3 kb and 2.5 kb in Ah_5.

Only two isolates viz. Ah_1 and Ah_3 harboured four plasmids. Though both of them shared a 21 kb plasmid, they were of two different profiles with respect to the remaining one. While the variant plasmids were of 6, 3 and 1.9 kb size in Ah_1, and 20, 5.5 and 5 kb in Ah_3.

The plasmid profiles were designated as 4(1), 4(2), 3(1), 3(2), 3(3), 2(1), 2(2) and 1(1), the first digit indicating the number of plasmids present in the profile.

Summary of plasmid numbers with their respective molecular weights are presented in the Table- 2.

Table--2. Summary of plasmids harboured by *Aeromonas hydrophila* and their size

Isolate No.	No. of plasmids	Plasmid profile No.	Size of plasmids (kb)
Ah_1	4	4(1)	21, 6, 3, 1.9
Ah_2	3	3(3)	21, 6, 3
Ah_3	4	4(2)	21, 20, 5.5, 5.0
Ah_4	2	2(1)	21, Mega Plasmid
Ah_5	3	3(2)	21, 3, 2.5
Ah_6	1	1(1)	21
Ah_7	1	1(1)	21
Ah_8	1	1(1)	21
Ah_9	1	1(1)	21
Ah_{10}	1	1(1)	21
Ah_{11}	1	1(1)	21
Ah_{12}	1	1(1)	21
Ah_{13}	3	3(1)	21, 2, 1
Ah_{14}	1	1(1)	21
Ah_{15}	3	3(1)	21, 2, 1
Ah_{16}	3	3(1)	21, 2, 1
Ah_{17}	1	1(1)	21
Ah_{18}	2	2(1)	21, Mega Plasmid
Ah_{19}	1	1(1)	21
Ah_{20}	1	1(1)	21
Ah_{21}	2	2(2)	21, 2
Ah_{22}	2	2(2)	21, 2

4.2. Antibiotic resistance

All the 22 strains of *Aeromonas hydrophila* under investigation were checked for their susceptibility against 20 antibiotics, by disc diffusion method. The characterization of strains as sensitive (S) and resistant (R) were done based on the size of the inhibition zones around each disc and antibiogram was prepared.

The resistance pattern of the isolates towards each of the 20 antimicrobial agents tested are shown in the Table- 3 and 4. Concurrently with the sharing of a major plasmid of 21 Kb by all the isolates, the resistance to 12 of the antibiotics tested were also similar among all the isolates. All isolates were resistant to 7 and sensitive to 5. They showed differences with respect to 8 antibiotics. The 7 antibiotics to which all the 22 isolates showed resistannce are ampicillin, amoxycillin, cephalexin, vancomycin, bacitracin, penicillin-G and lincomycin. The sensitive antibiotics were ofloxacin, nitrofurazone, gentamycin, chloramphenicol and pefloxacin.

Differential resistance was shown by these isolates only to 8 of the antibiotics tested. These antibiotics were neomycin, cefaclor, carbenicillin, norfloxacin, oxytetracycline, erythromycin, amikacin and doxycycline. The percentage of resistant isolates to the above antibiotics were, 90.90% , 86.36% , 81.81% , 72.72% , 68.18% , 68.18% , 50% and 36.36% respectively. Information on the plasmid profile and antibiotics resistance to each of the isolates are summarized ion Table--5, enabling comparison of the antibiotic resistance of different plasmid profile groups. Both the isolates with four plasmids each was hundred percent similar in their antibiogram. In the group with three plasmids each, the antibiogram pattern varied with the plasmid profile with respect to 5 antibiotics (A, K, Cb, Do, E and Kf). While the profile no. 3(1) was resistant to Ak, Cb, E, Kf and N, the profile no. 3(2) was sensitive to Ak and N. However, The profile no. 3(3) was sensitive to Ak,Cb, Do and E. The group with two plasmids showed variations in the resistance of Ak, Do, Kf and O. While one of the two isolates of profile 2(1) was resistant to Do and Kf and sensitive to Ak and O, the other isolates were resistant to Ak and sensitive to Do, Kf and O. The two isolates of profile no. 2(2) were different only in respect of two antibiotics viz. Kf and O only. The single plasmid isolates were similar in their antibiogram pattern except for Ak, Do, E, Kf, O and Cb.

Table 3. Antibiogram of *Aeromonas hydrophila* isolates(Ah_1-Ah_{11})

Sl.No..	ANTIBIOTICS	Symbol	Ah_1	Ah_2	Ah_3	Ah_4	Ah_5	Ah_6	Ah_7	Ah_8	Ah_9	Ah_{10}	Ah_{11}
1	AMIKACIN	Ak	R	R	R	S	S	S	S	S	S	R	R
2	AMOXYCILLIN	Am	R	R	R	R	R	R	R	R	R	R	R
3	AMPICILLIN	A	R	R	R	R	R	R	R	R	R	R	R
4	BACITRACIN	B	R	R	R	R	R	R	R	R	R	R	R
5	CARBENICILLIN	Cb	R	R	R	R	S	R	R	R	R	R	S
6	CEFACLOR	Kf	R	R	R	R	R	R	R	R	R	R	R
7	CEPHALEXIN	Cp	R	R	R	R	R	R	R	R	R	R	R
8	CHLORAMPHENICOL	C	S	S	S	S	S	S	S	S	S	S	S
9	DOXYCYCLINE	Do	S	S	S	R	S	R	R	S	S	S	S
10	ERYTHROMYCIN	E	R	R	R	R	S	R	S	S	S	S	R
11	GENTAMICIN	G	S	S	S	S	S	S	S	S	S	S	S
12	LINCOMYCIN	L	R	R	R	R	R	R	R	R	R	R	R
13	NEOMYCIN	N	R	R	R	R	R	R	R	R	R	R	R
14	NITROFURAZONE	Nr	S	S	S	S	S	S	S	S	S	S	S
15	NORFLOXACIN	Nx	S	R	S	R	R	R	R	R	R	R	R
16	OFLOXACIN	Of	S	S	S	S	S	S	S	S	S	S	S
17	OXYTETRACYCLINE	O	R	R	R	S	R	R	R	R	R	S	R
18	PEFLOXACIN	Pef	S	S	S	S	S	S	S	S	S	S	S
19	PENICILLIN-G	P	R	R	R	R	R	R	R	R	R	R	R
20	VANCOMYCIN	Va	R	R	R	R	R	R	R	R	R	R	R

Table 4. Antibiogram of *Aeromonas hydrophila* isolates (Ah_{12}-Ah_{22})

SL.NO.	ANTIBIOTICS	SYMBOL	Ah_{12}	Ah_{13}	Ah_{14}	Ah_{15}	Ah_{16}	Ah_{17}	Ah_{18}	Ah_{19}	Ah_{20}	Ah_{21}	Ah_{22}
1	AMIKACIN	Ak	R	S	S	S	S	R	R	R	S	R	R
2	AMOXYCILLIN	Am	R	R	R	R	R	R	R	R	R	R	R
3	AMPICILLIN	A	R	R	R	R	R	R	R	R	R	R	R
4	BACITRACIN	B	R	R	R	R	R	R	R	R	R	R	R
5	CARBENICILLIN	Cb	R	R	R	R	R	S	R	S	R	R	R
6	CEFACLOR	Kf	R	R	R	R	S	R	S	R	R	S	R
7	CEPHALEXIN	Cp	R	R	R	R	R	R	R	R	R	R	R
8	CHLORAMPHENICOL	C	S	S	S	S	S	S	S	S	S	S	S
9	DOXYCYCLINE	Do	S	R	R	R	S	R	S	S	S	R	S
10	ERYTHROMYCIN	E	S	R	S	R	R	R	R	R	R	R	R
11	GENTAMICIN	G	S	S	S	S	S	S	S	S	S	S	S
12	LINCOMYCIN	L	R	R	R	R	R	R	R	R	R	R	R
13	NEOMYCIN	N	R	S	S	R	R	R	R	R	R	R	R
14	NITROFURAZONE	Nr	S	S	S	S	S	S	S	S	S	S	S
15	NORFLOXACIN	Nx	S	R	S	R	R	R	S	R	S	R	R
16	OFLOXACIN	Of	S	S	S	S	S	S	S	S	S	S	S
17	OXYTETRACYCLINE	O	S	R	R	R	R	R	S	S	S	R	S
18	PEFLOXACIN	Pef	S	S	S	S	S	S	S	S	S	S	S
19	PENICILLIN-G	P	R	R	R	R	R	R	R	R	R	R	R
20	VANCOMYCIN	Va	R	R	R	R	R	R	R	R	R	R	R

Table- 5. Plasmid DNA profiles and antibiotic resistance pattern of *Aeromonas hydrophila* isolates (Ah_1-Ah_{22})

Isolate No.	Plasmid profile group	Size of plasmids (kb)	Antibiotics
Ah_1	4(1)	21, 6, 3, 1.9	Ak, Am, A, B, Cb, Kf, Cp, E, L, N, O, P, Va.
Ah_2	3 (3)	21, 6, 3	Ak, Am, A, B, Cb, Kf, Cp, E, L, N, Nx, O, P,Va.
Ah_3	4 (2)	21, 20, 5.5, 5.0	Ak, Am, A, B, Cb, Kf, Cp, E, L,N,O, P, Va.
Ah_4	2 (1)	21, Mega plasmid	Am, A, B, Cb, Kf, Cp, Do, E, L, N, Nx, P, Va.
Ah_5	3 (2)	21, 3,2.5	Am, A, B, Kf, Cp, L, N, Nx, O, P, Va.
Ah_6	1 (1)	21	Am, A, B, Cb, Kf, Cp, Do, E, L, N, Nx, O, P, Va.
Ah_7	1 (1)	21	Am, A, B, Cb, Kf, Cp, Do, L, N, Nx, O, P, Va.
Ah_8	1 (1)	21	Am, A, B, Cb, Kf, Cp, L, N, Nx, O, P, Va.
Ah_9	1 (1)	21	Am, A, B, Cb, Kf, Cp, L, N, Nx, O, P, Va.
Ah_{10}	1 (1)	21	Ak, Am, A, B, Cb, Kf, Cp, L, N, Nx, P, Va.
Ah_{11}	1 (1)	21	Ak, Am, A, B, Kf, Cp, E, L, N, Nx, O, P, Va.
Ah_{12}	1 (1)	21	Ak, Am, A, B, Cb, Kf, Cp, L, N,P,Va.
Ah_{13}	3 (1)	21, 2, 1	Am, A, B, Cb, Kf, Cp, Do, E, L, Nx, O, P, Va.
Ah_{14}	1 (1)	21	Am, A, B, Cb, Kf, Cp, Do, L,O,P,Va.
Ah_{15}	3 (1)	21, 2, 1	Am, A, B, Cb, Kf, Cp, Do, E, L, N, Nx, O, P,Va.
Ah_{16}	3 (1)	21, 2, 1	Am, A, B, Cb, Cp, E, L, N, Nx,O,P,Va.
Ah_{17}	1 (1)	21	Ak, Am, A, B, Kf. Cp. Do, E, L, N, Nx, O, P,Va.
Ah_{18}	2 (1)	21, Mega plasmid	Ak, Am, A, B, Cb, Cp, E, L, N, Nx, P, va
Ah_{19}	1 (1)	21	Ak,Am,A,B,Kf,Cp,E,L,N,Nx,P,Va.
Ah_{20}	1 (1)	21	Am,A,B,Cb,Kf,Cp,E,L,N,P,Va.
Ah_{21}	2 (2)	21, 2	Ak,Am,A,B,Cb,Cp,E,L,N,NxO,P,Va.
Ah_{22}	2 (2)	21, 2	Ak,Am,A,B,Cb,Kf,Cp,E,L,N,Nx,P,Va.

4.3. Cellular protein profile

4.3.1. NATIVE-PAGE profile

Analysis of cellular proteins extracted from all the isolates was carried out by using the NATIVE-PAGE technique. The protein profiles thus resolved are presented in the Fig- 4 and 5.

The protein profiles of all twenty two isolates were compared to find out specific patterns if any. A total of 11 different proteins were expressed by them, of which only six were present in all the isolates. The number of protein bands in an individual isolate ranged between 6 and 10. Eleven of the isolates (Ah_3, Ah_4, Ah_5, Ah_6, Ah_7, Ah_8, Ah_{11}, Ah_{12}, Ah_{13}, Ah_{15} and Ah_{19}) expressed only commonly shared six proteins and therefore, had a similar profile. The other 11 isolates expressed the remaining 5 proteins in varying combinations. One protein was additionally present in 8 of the remaining isolates making the number of bands to seven, the one protein additionally expressed in Ah_1 and Ah_2 were different from that was expressed in Ah_9, Ah_{10}, Ah_{17}, Ah_{20}, Ah_{21} and Ah_{22}, resulting in two different proteins profile types. The additional protein of Ah_1 and Ah_2 were not present in any other isolates. Ah_{16} had two additional proteins not expressed by any of the other isolates. The maximum number of proteins were expressed in Ah_{17} and Ah_{18} with 4 additional proteins of similar size resulting in similar protein profile. The uniformity of cellular protein profile was limited to the six low molecular size proteins expressed by all of them.

4.3.2. SDS-PAGE

Cellular proteins extracted from all the isolates were resolved through SDS-PAGE. The cellular protein fingerprint pattern thus generated are presented in the Fig- 6 and 7.

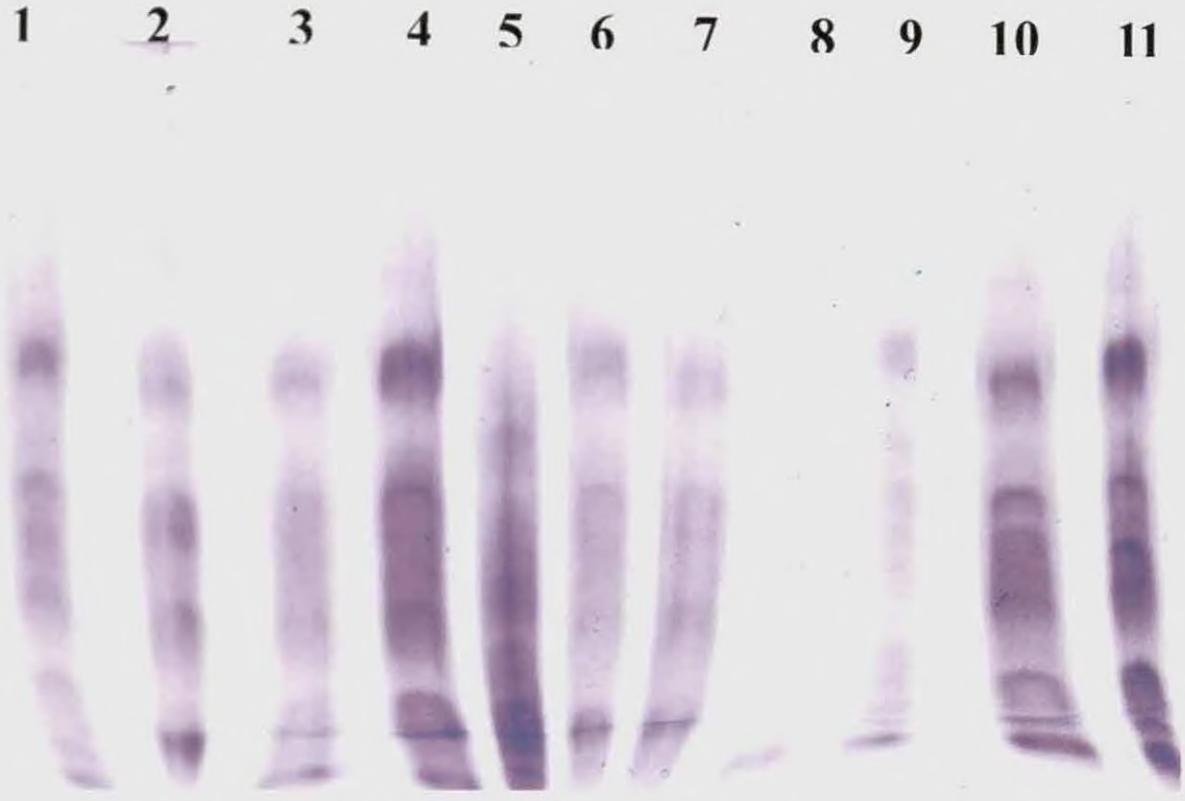


Fig :4

Cellular Protein Profile of *Aeromonas hydrophila* isolates resolved through NATIVE-PAGE electrophoresis.

Lane 1-11: Ah_1, Ah_2, Ah_{13}, Ah_{15}, Ah_3, Ah_5, Ah_{16}, Ah_4, Ah_7, Ah_8, Ah_6

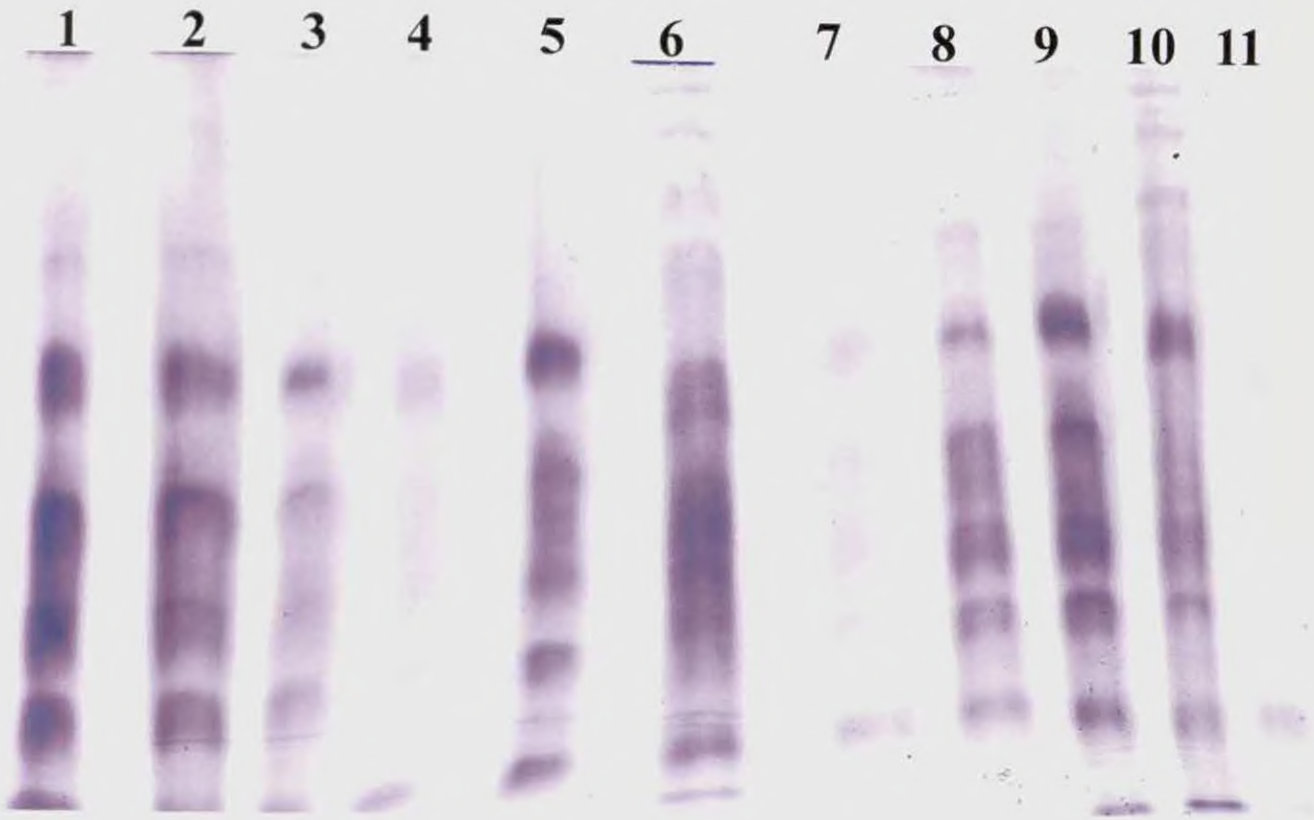


Fig :5

Cellular Protein Profile of *Aeromonas hydrophila* isolates resolved through NATIVE-PAGE electrophoresis

Lane 1-11: Ah_9 , Ah_{10} , Ah_{11}, Ah_{12}, Ah_{17}, Ah_{18}, Ah_{19}, Ah_{20}, Ah_{21}, Ah_{22}, Ah_{14}

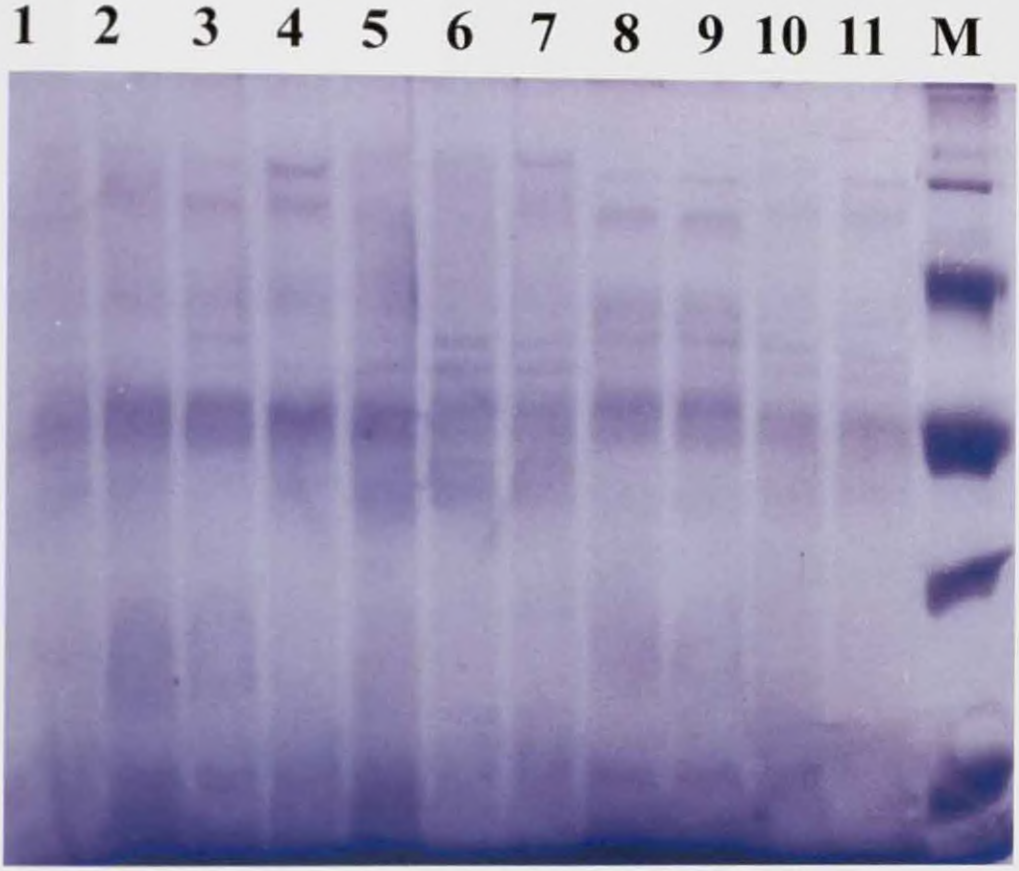


Fig :6

Cellular Protein Profile of *Aeromonas hydrophila* isolates resolved through SDS-PAGE electrophoresis

Lane 1-11: Ah_1, Ah_2, Ah_3, Ah_4, Ah_5, Ah_6, Ah_7, Ah_8, Ah_9, Ah_{10}, Ah_{11},

Lane M : Marker

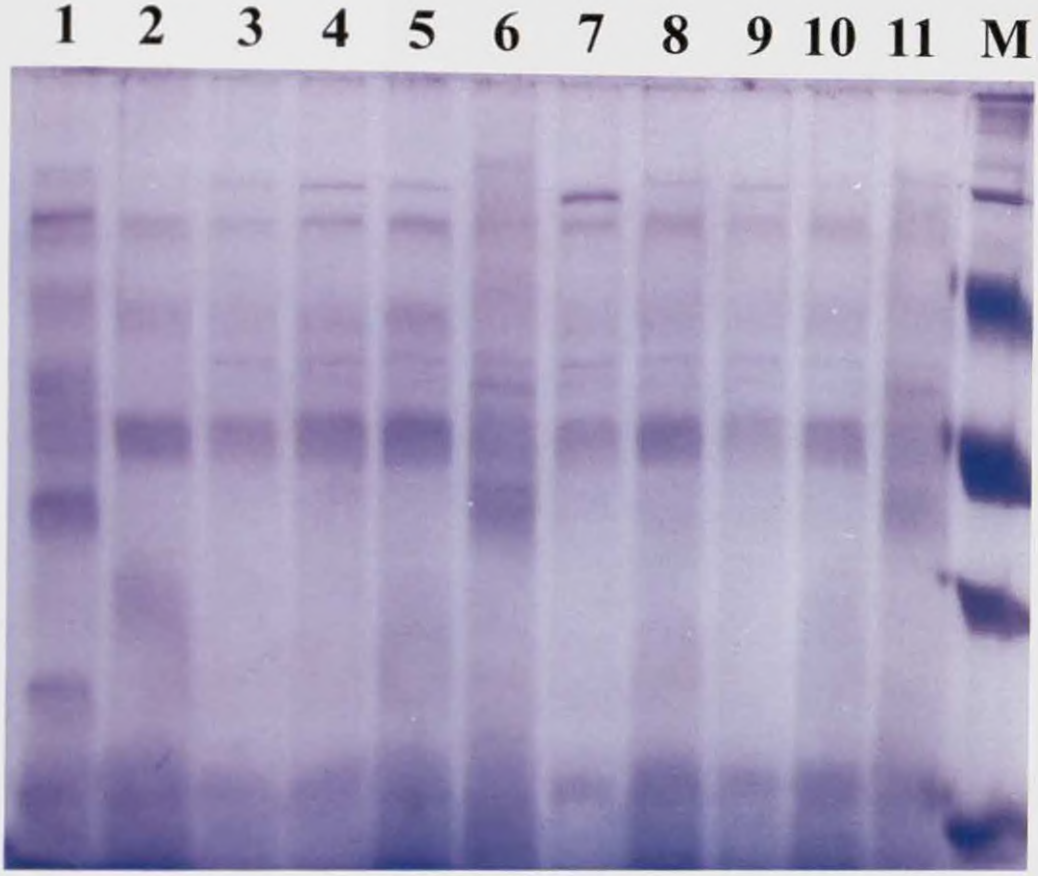


Fig :7

Cellular Protein Profile of *Aeromonas hydrophila* isolates resolved through SDS-PAGE electrophoresis

Lane 1-11: Ah_{12}, Ah_{13}, Ah_{14}, Ah_{15}, Ah_{16}, Ah_{17}, Ah_{18}, Ah_{19}, Ah_{20}, Ah_{21}, Ah_{22}

Lane M : Marker

The SDS-PAGE profile of cellular proteins of all twenty-two strains were compared to find out specific patterns if any. A total of 14 different protein bands were observed from these isolates and their molecular sizes ranged from 18 KDa to >100 KDa. As can be seen from the Table--6, three of them with molecular weights of 48 KDa, 43 KDa and 18 KDa were expressed by all isolates while 11 were differentially expressed. The differentially expressed proteins were >100 KDa, 100 KDa, 97 KDa, 90 KDa, 80 KDa, 66 KDa, 60 KDa, 54 KDa, 35 KDa, 30 KDa and 19 KDa, the details of which are presented below.

Table- 6. Summary of Cellular Protein Profile resolved through SDS-PAGE

Isolate	No. of bands	Molecular weight (KDa) of the protein bands
Ah_1	8	>100,100,-,-,-,66,-,-, 48,43,-,30,19,18
Ah_2	8	>100,100,-,90,-,66,-,-, 48, 43,-,-,19,18
Ah_3	8	-,-,97,90,-,66,60,54,48, 43,-,-,-,18
Ah_4	7	-,-,97,90,-,66,-,54, 48, 43,-,-,-,18
Ah_5	8	>100,100,-,-,-,66,-,-,48,43,35,30,-,18
Ah_6	10	>100,100,97,-,-,-,60,54,48, 43,35,30, -,18
Ah_7	7	>100,100,-,-,-,-,60,54,48,43,-, -,-,18
Ah_8	8	-,-,97,90,-,66,60,54, 48, 43, -,-,-,18
Ah_9	8	-,-,97,90,-,66,60, 54, 48, 43,-,-,-,18
Ah_{10}	6	-,100,-, -,-, -,60,54,48, 43, -,-,-,18
Ah_{11}	6	-,-,97,-,-,-,60,54, 48, 43,-,-,-,18
Ah_{12}	14	>100, 100,97,90,80,66,60, 54,48,43,35,30,19,18
Ah_{13}	5	-,-,-,90,-,66,-,-,48,43,-, -,-,18
Ah_{14}	7	-,-,97,90,-,-,60,54,48,43, -,-,-,18
Ah_{15}	8	-,-,97,90,-,66,60, 54, 48, 43, -,-, -,18
Ah_{16}	8	-,-,97,90,-,66,60,54, 48, 43, -,-,-,18
Ah_{17}	10	>100, 100,-,-,-,-, 60,54,48,43,35, 30,19,18
Ah_{18}	7	-,-,-,90, -, 66,60,54, 48, 43, -,-,-,18
Ah_{19}	8	-,-,97,90,-,-,60, 54,48, 43, -,-,19,18
Ah_{20}	7	-,-,97,90,-,-,60,54, 48, 43, -, -,-,18
Ah_{21}	8	-,-,97,90,-,-,60,54,48, 43,-,-,19,18
Ah_{22}	11	>100,100,-,90,-,-,60,54, 48, 43, 35, 30,19,18

The number of proteins present in a single isolate varied from 5 to 14. As can be seen from the Table--6, while Ah_{12} had all the 14 protein bands , Ah_{22} had only 11. Although both Ah_6 and Ah_{17} had ten each, they were different with respect to 97 and 19 KDa proteins. Eight bands were expressed by ten isolates as against 7 bands by 5 of the isolates. Though 6 bands were present in two isolates namely Ah_{10} and Ah_{11}, there were differences with respect to the 100 and 90 KDa proteins. The minimum number of 5 bands was expressed only by one isolate viz. Ah_{13}. The SDS-PAGE resolved protein profile revealed more diversity compared to the NATIVE-PAGE resolved protein profile.

4.5. RAPD-PCR

RAPD was carried out using plasmid DNA as template,1,2, 3 and 4 banded plasmids. Plasmid DNA from seven isolates namely one from each of the plasmid profile group were selected for use as RAPD template. Details of the selected isolates are as given in Table- 7.

Table- 7. Isolates Selected for RAPD

Sl. No.	Plasmid profile	Isolate	Plasmid profile no.
1	21, 6, 3, 1.9 Kb	Ah_1	4(1)
2	21, 20, 5.5, 5 Kb	Ah_3	4(2)
3	21, 2, 1 Kb	Ah_{13}	3(1)
4	21, 3, 2.5 Kb	Ah_5	3(2)
5	21, >100 Kb	Ah_4	2(1)
6	21, 2 Kb	Ah_{21}	2(2)
7	21Kb	Ah_7	1(1)

The optimum conditions for the random primed PCR amplification of plasmid DNA were as follows. Concentration of $MgCl_2$ in assay buffer was optimized as 1.5mM. Taq polymerase and dNTP'S were

standardized at 1.25 units and 200µM respectively. The annealing condition in the PCR cycle was found to be optimum at 37^0C.

Four decamer primers i.e.OPA-01 to OPA-04 were used to amplify the plasmid DNA from each isolates. A total of 93 different amplicons (amplified fragments) were produced from these 7 isolates using the four primers, which appeared as distinct bands on agarose gel after electrophoresis. The molecular weight of these amplicons ranged from 0.20Kb to 4.5Kb as determined from the relative mobility of marker DNA viz. λ DNA cut with Hind III/ EcoRI. The RAPD patterns generated by each of the four primers are as follows.

OPA-01

This primer produced 26 different amplicons (amplified fragments) in total. Majority of the amplified fragments were polymorphic. Eight amplicons were shared by all isolates while eighteen amplicons showed variation between isolates. The amplicons shared by all isolates were of 1.3Kb, 0.90Kb, 0.83Kb, 0.80Kb, 0.50Kb, 0.45Kb, 0.40Kb and 0.35Kb. The fragment of 4.2Kb and 0.42Kb was present only in strain Ah_{13} while the fragment of 2.0Kb, 1.9Kb and 1.5Kb was present only in Ah_1. The fragment of 3.0Kb was shared by Ah_1 and Ah_{21}. The fragment of 1.2Kb was shared by Ah_3 and Ah_{13} while fragment of 0.95Kb was shared by Ah_{13} and Ah_{21}. The fragment of 0.94Kb was shared by all except Ah_5 and fragment of 0.75Kb was shared by all except Ah_7 while fragment of 0.42Kb was present in all except Ah_4. Fragment of 3.0Kb was present in all except Ah_3 and Ah_{13} while fragment of 1.0Kb was shared by all except Ah_5 and Ah_{17} and fragment of 0.56Kb was shared by all except Ah_5 and Ah_{13}. The frequency of each of the amplicons produced by OPA-01 are presented in the Table--8 and the frequency ranges from 0.1250 to 1.0000. The Fig-8 presents the RAPD fingerprints of isolates generated by OPA-01 primer.

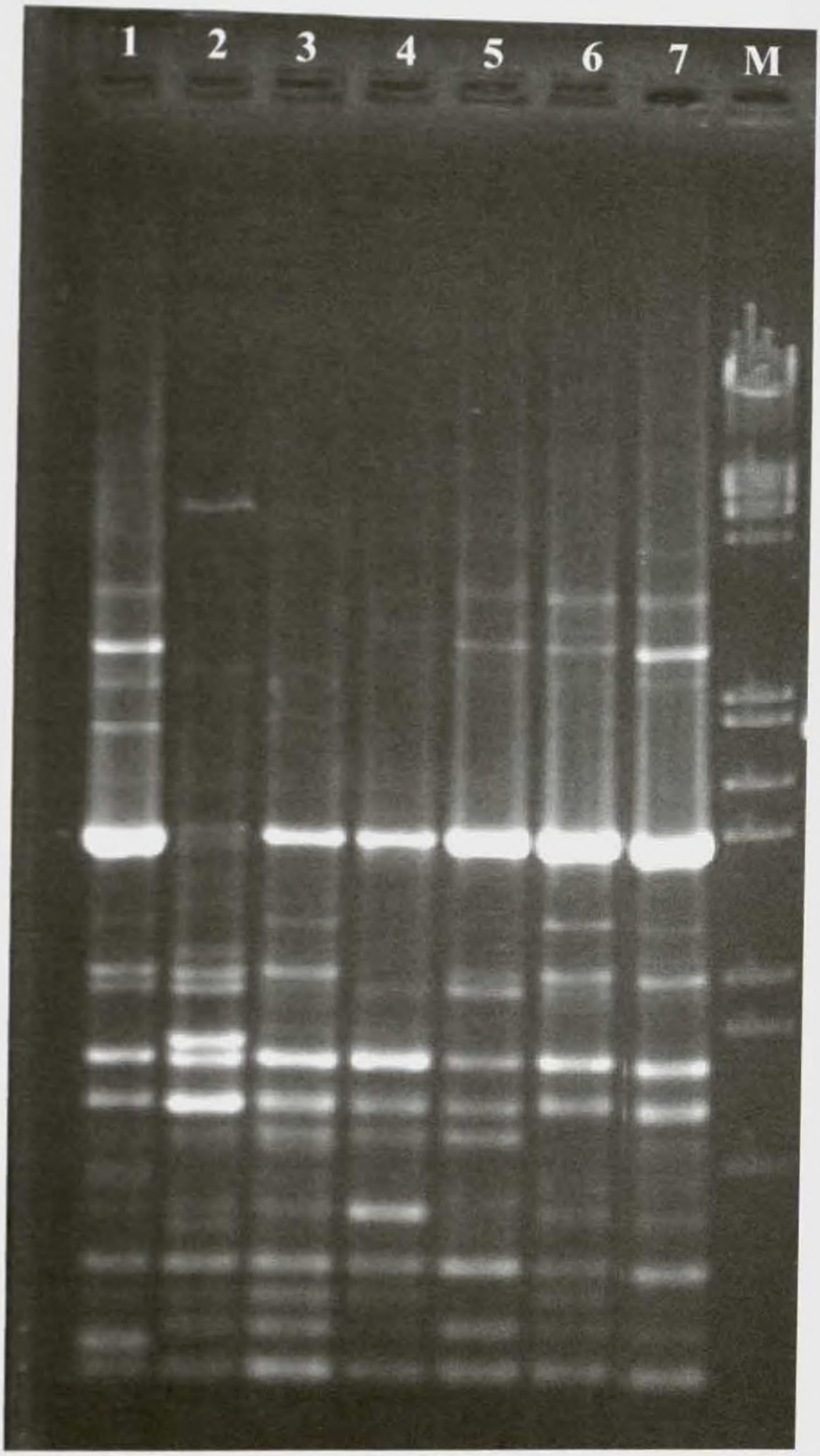


Fig. 8

RAPD patterns generated from plasmid DNA of *Aeromonas hydrophila* using OPA-01
Lane 1-7 : Ah_1, Ah_{13}, Ah_3, Ah_5, Ah_4, Ah_7, Ah_{21}
Lane M : Marker

Table 8. Amplified plasmid DNA fragment frequency across the seven plasmid profile groups

Primer / Amplicon	OPA-01	OPA-02	OPA-03	OPA-04
1	0.1429	0.7143	0.1429	0.2857
2	0.2857	0.1429	0.8571	0.1429
3	0.7143	0.2857	0.1429	0.5714
4	0.5714	0.7143	0.8571	0.5714
5	0.5714	0.2857	0.1429	0.7143
6	0.1429	0.7143	0.1429	0.1429
7	0.1429	0.7143	0.2857	0.7143
8	0.1429	0.2857	0.1429	0.7143
9	1.0000	0.5714	0.8571	0.1429
10	0.2857	0.8571	0.8571	0.2857
11	0.7143	0.7143	0.1429	0.5714
12	0.2857	0.7143	0.4286	0.8571
13	0.8571	0.5714	1.0000	0.4286
14	1.0000	0.4286	0.5714	0.2857
15	0.2857	0.5714	0.4286	0.7143
16	0.4286	0.7143	0.8571	0.1429
17	1.0000	0.5714	0.7143	0.1429
18	1.0000	0.1429	0.2857	0.1429
19	0.8571	0.8571	0.1429	0.1429
20	0.5714	0.7143		1.0000
21	1.0000			0.5714
22	0.1429			0.8571
23	1.0000			1.0000
24	0.8571			0.5714
25	1.0000			0.8571
26	1.0000			0.8571
27				1.0000
28				1.0000

OPA-02

Twenty amplicons were generated by OPA-02 and all were within the range of 0.60 Kb to 4.2 Kb. Of these, the fragment of 0.80 Kb was present in Ah_{13} while fragment of 3.0 Kb was present only in Ah_5. The fragment of 2.5 Kb was shared by Ah_5 and Ah_{21} while fragment of 2.30 Kb was shared by Ah_1 and Ah_5 and fragment of 1.70 Kb was shared by Ah_3 and Ah_5. Fragment of 0.70 Kb was shared by all except Ah_{13}. The amplicons of 0.60 Kb and 1.80 Kb was shared by all except Ah_3 and Ah_5 while amplicon of

0.95 Kb and 1.20 Kb was shared by all except Ah_3 and Ah_{13}. The amplicon of 4.20 Kb and 1.90 Kb was present in all except Ah_5 and Ah_{13}. The fragment of 1.50 kb was shared by all except Ah_3 Ah_5 and Ah_{13}. The frequency of amplicons ranged from 0.1250 to 0.8750. The Fig-9 presents the RAPD fingerprints of isolates generated by OPA-02 primer.

OPA-03

Total number of amplicons generated by OPA-03 were nineteen and they were within the range of 4.90 Kb to 0.30 Kb. The Fig- 10 presents the RAPD fingerprints of isolates generated by OPA-03 primer and frequency of amplicons ranged from 0.1250 to 1.0000. The fragments of 4.90 Kb, 3.40Kb, 2.10 Kb,1.20 Kb and 0.30 Kb were present in isolate Ah_{13} only. The fragment of 2.0 Kb was present in Ah_1 while fragment of 1.70Kb was present in Ah_5 only The fragment of 1.40 Kb and 1.0 Kb was shared by all isolates. The fragment of 3.45 Kb and 0.83Kb were shared by all isolates except Ah_3 and Ah_1 respectively The fragment of 3.30 Kb was shared by all except Ah_1 and Ah_{13} while fragment of 1.50 Kb was shared by all except Ah_3 andAh_{17}. The fragment of 1.80 Kb was shared by Ah_5 and Ah_{13} while fragment of 0.60 Kb was present in Ah_3 and Ah_{21} only. The fragment of 1.10 Kb was shared by Ah_3, Ah_5 and Ah_{13} while fragment of 0.90 Kb was shared by Ah_4,Ah_5 and Ah_7. The fragment of 0.95 Kb was shared by Ah_4, Ah_5, Ah_7, Ah_{17} and Ah_{21}.

Opa-04

Twenty eight amplicons were generated by the primer OPA-04 within the range of 20.0 Kb to 0.30 Kb and RAPD fingerprints of isolates are presented in the Fig- 11. The amplicons of 0.94 Kb, 0.83 Kb, 0.40 Kb and 0.30 Kb were shared by all isolates. The fragment of 1.90 Kb, 0.65 Kb and 0.60 Kb were shared by all except Ah_{13} while fragment of 0.85 Kb was shared by all except Ah_1 The fragment of 2.0 Kb and 1.50 Kb were present in all isolates except Ah_3 and Ah_{13} while fragments of 1.25 Kb was shared by all except Ah_1 and Ah_{13} and fragment of 1.40 Kb was present in all isolates

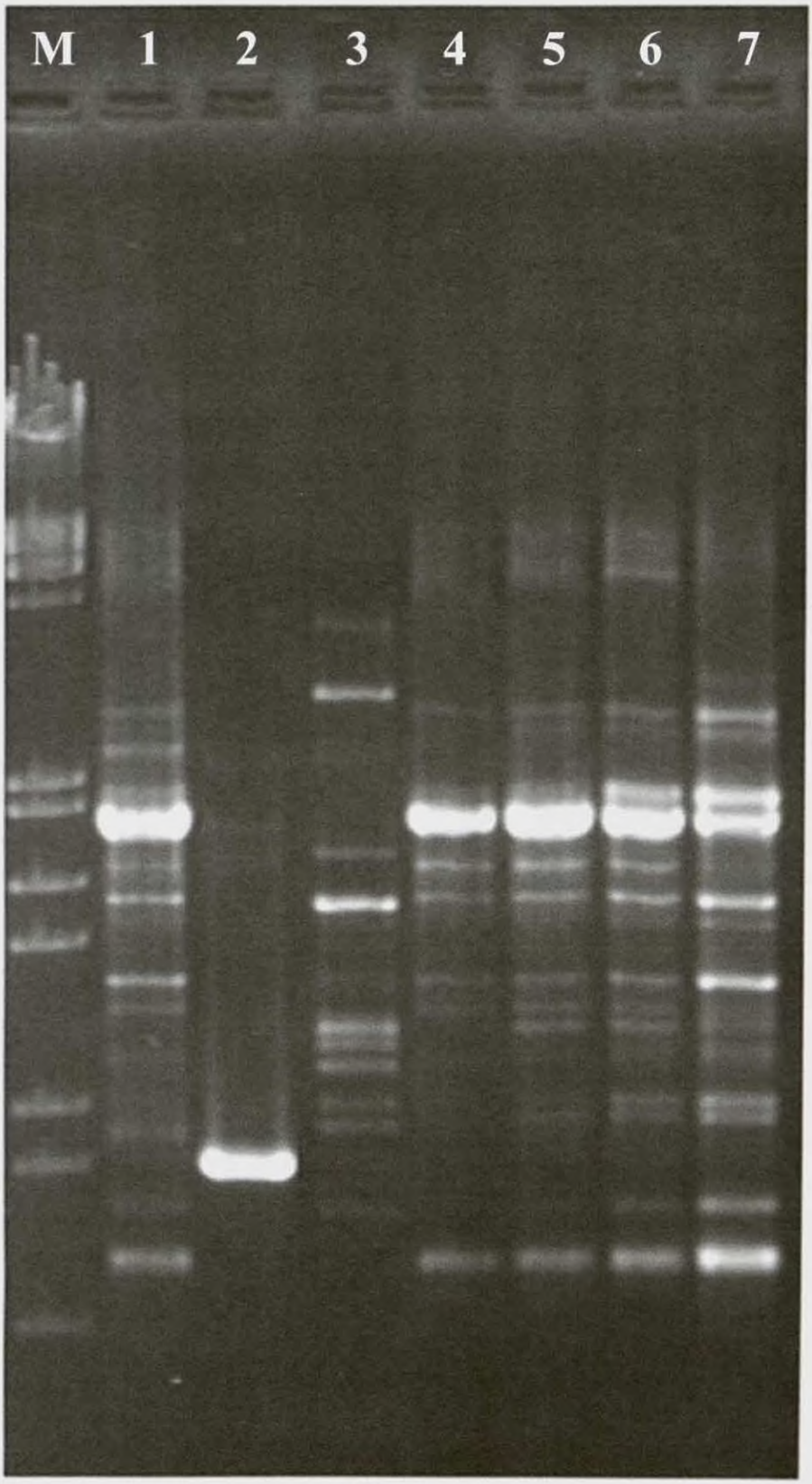


Fig. 9

RAPD patterns generated from plasmid DNA of *Aeromonas hydrophila* using OPA-02
Lane 1-7 : Ah_1, Ah_{13}, Ah_3, Ah_5, Ah_4, Ah_7, Ah_{21}
Lane M : Marker

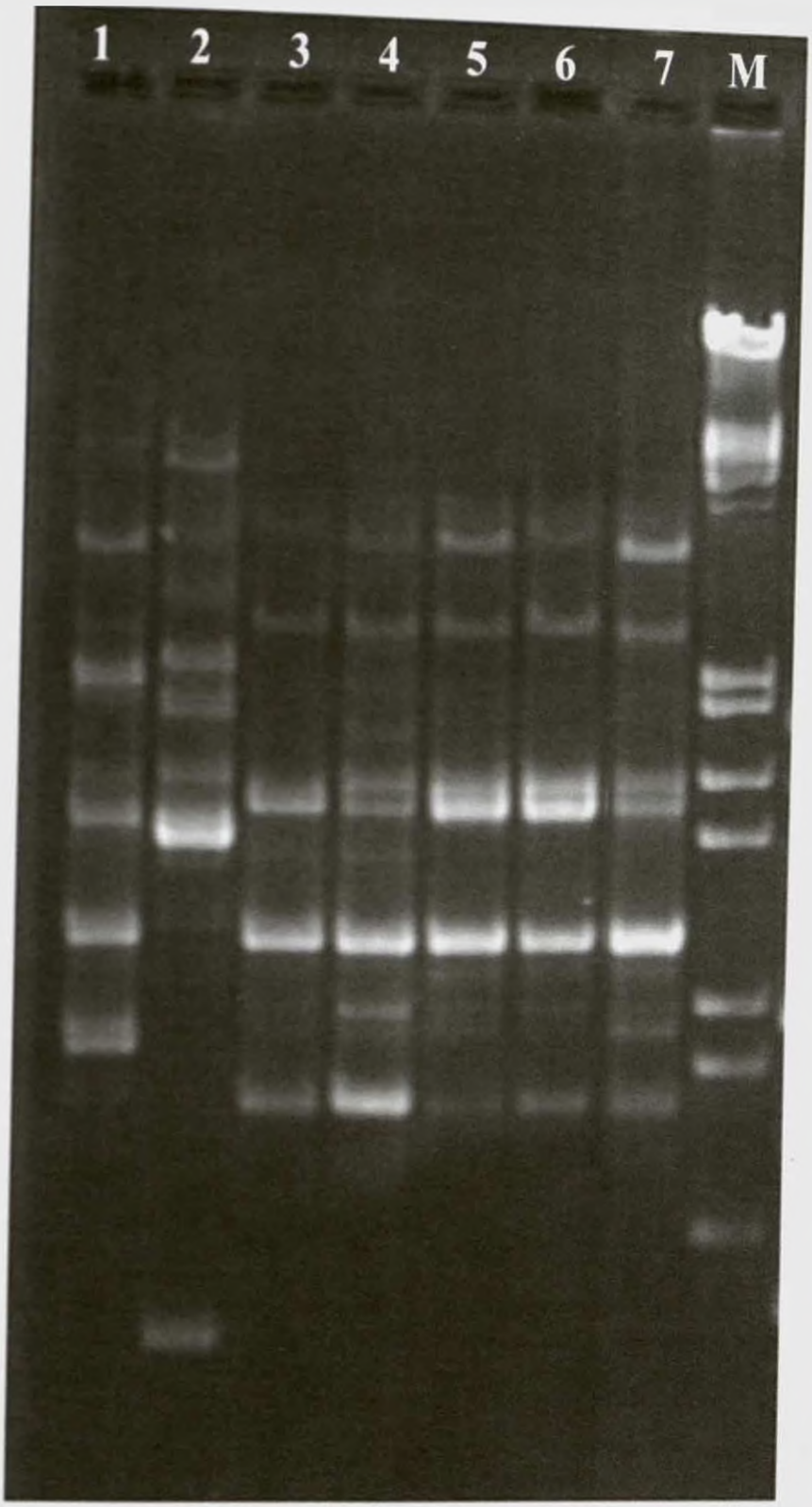


Fig. 10

RAPD patterns generated from plasmid DNA of *Aeromonas hydrophila* using OPA-03
Lane 1-7 : Ah_1, Ah_{13}, Ah_3, Ah_5, Ah_4, Ah_7, Ah_{21}
Lane M : Marker

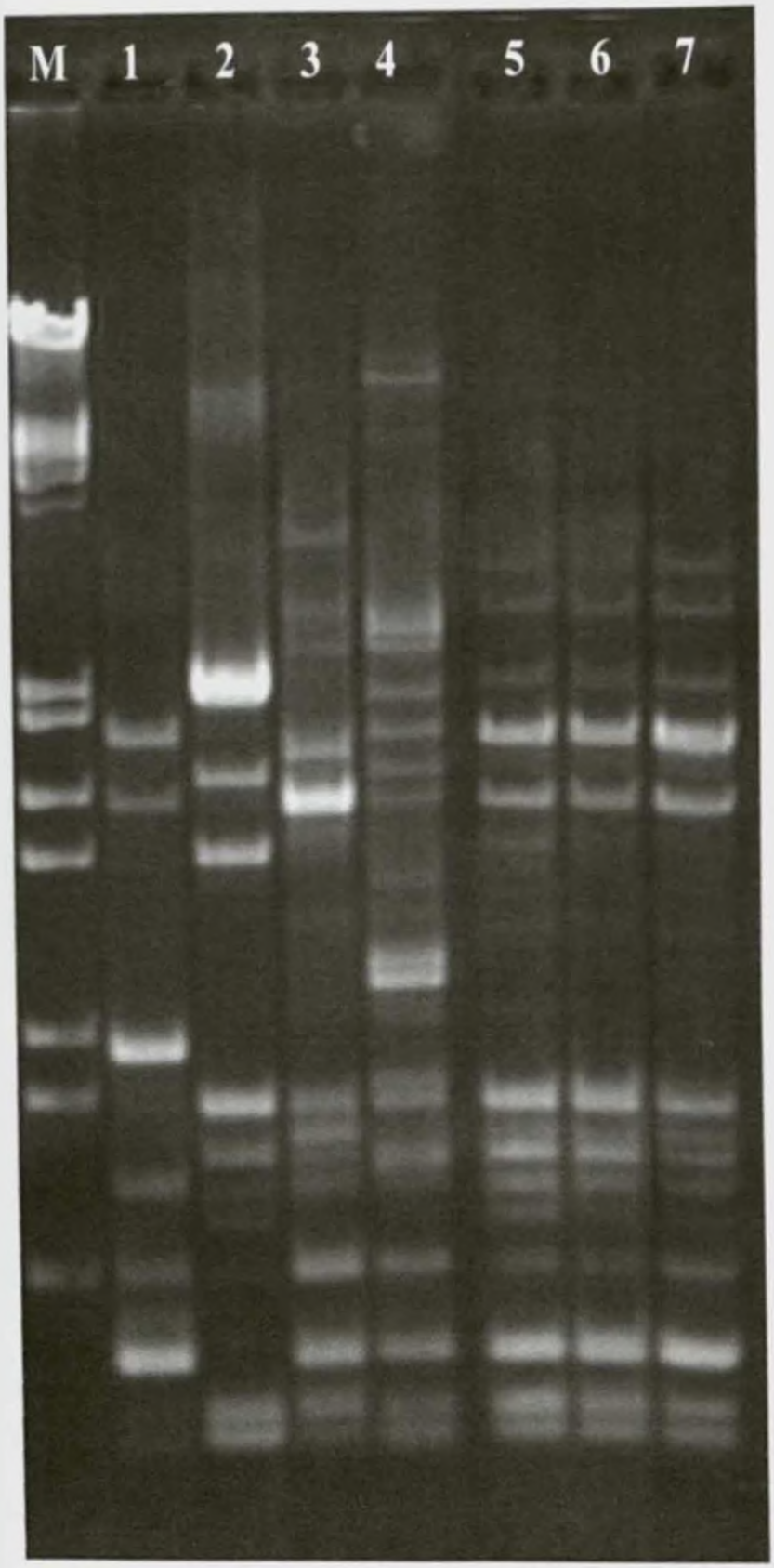


Fig. 11

RAPD patterns generated from plasmid DNA of *Aeromonas hydrophila* using OPA-04
Lane 1-7 : Ah_1, Ah_{13}, Ah_3, Ah_5, Ah_4, Ah_7, Ah_{21}
Lane M : Marker

except Ah_1 and Ah_3. The fragment of 1.0 Kb was shared by only Ah_1 and fragment of 2.50 Kb was present in Ah_3 only while fragments of 4.20 Kb, 1.45 Kb, 1.20 Kb, 1.15 Kb and 0.95 Kb was present in Ah_5 only. The fragment of 20.0 Kb was shared by Ah_5 and Ah_{13}. The fragment of 1.30 Kb was present in all except Ah_1, Ah_3, and Ah_5. The frequency of amplicons ranged from 0.1250 to 1.0000.

Polymorphism, *Similarity index, Genetic distance and Phylogenetic relationship*

The data on the random amplified DNA fragments produced from the plasmid DNA of the 7 plasmid profile groups were analyzed by the POPGENE 32 software and the resulting estimates are as below.

Polymorphism of the random amplified DNA fragments was quite apparent among different plasmid profiles. RAPD fingerprint pattern was unique for each of plasmid profile group with all four primers. Comparison of the fragments at each of the 93 loci indicated that 80 fragments were polymorphic. Out of 13 non-polymorphic loci 8, 1 and 4 were of OPA-01, OPA-03 , OPA-04 respectively. The POPGENE analysis indicated that the overall polymorphism was 86.02%.

The average similarity index between different plasmid profile groups within the species, considering the all fragments resulting from all the primers estimated as Nei's original measures of genetic identity and genetic distance between the seven plasmid profile groups are shown in Table- 9. Content of Table-9 indicates that coefficient of genetic identities ranged from 0.4301 to 0.9355.

Dendogram in the phylogram form depicting the phylogenetic relatedness among the isolates having different plasmid profiles, generated

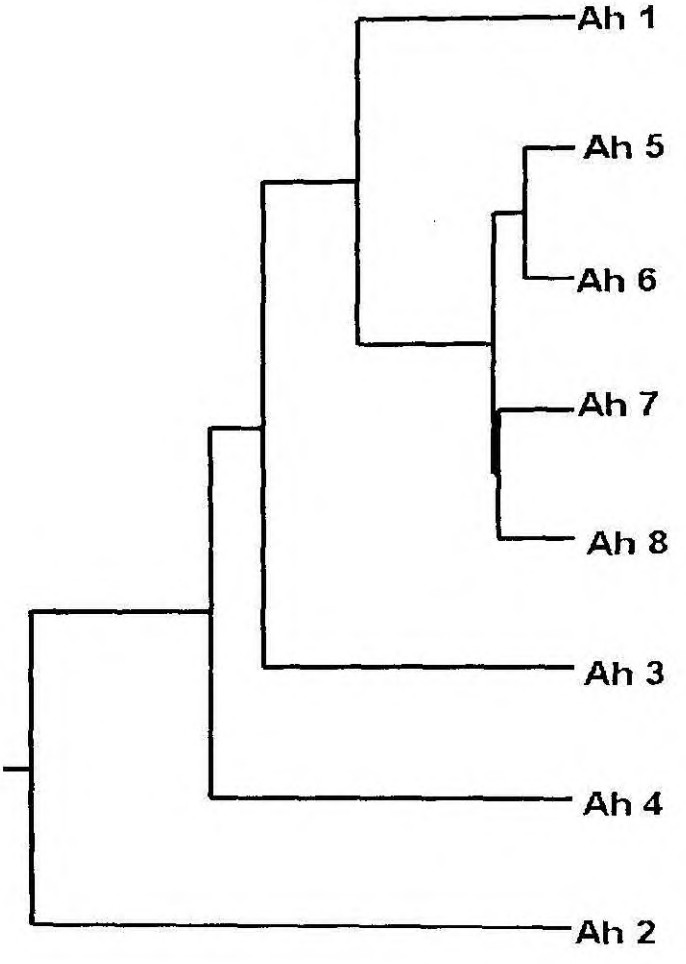
from the RAPD data by unweighted pair group method using arithmetic average (UPGMA) is presented in Fig- 12.

Table 9. Nei's original measures of Genetic Identity and Genetic Distance between the different plasmid profile groups

Pop ID	4(1)	3(1)	4(2)	3(2)	2(1)	1(1)	2(2)
4(1)	****	0.4301	0.6344	0.5806	0.7312	0.7312	0.7634
3(1)	0.8437	****	0.4946	0.4839	0.5269	0.4839	0.4731
4(2)	0.4551	0.704	****	0.6022	0.6667	0.6667	0.6774
3(2)	0.5436	0.7259	0.5072	****	0.6129	0.6129	0.6237
2(1)	0.3131	0.6408	0.4055	0.4895	****	0.9355	0.8817
1(1)	0.3131	0.7259	0.4055	0.4895	0.0667	****	0.9032
2(2)	0.2699	0.7484	0.3895	0.4722	0.1259	0.1018	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Fig. 12. Dendrogram based on the RAPD data of plasmid DNA of *Aeromonas hydrophila* isolates showing genetic relatedness among them.



5. Discussion

DISCUSSION

The present work was undertaken with the main aim of elucidating the molecular genetic diversity at the level of plasmid DNA, cellular proteins as well as antibiotic resistance in the field strains of *Aeromonas hydrophila* isolated from aquaculture systems.

Plasmids, the extrachromosomal DNA of bacteria, are the genetic material coding for a number of factors responsible for resistance to antimicrobial drugs. Since plasmids are easily transferable from bacterium to bacterium through conjugation, the field strains can undergo sudden changes in their plasmid carriage causing diversity in plasmid profile and the resultant antibiotic resistance pattern.

As postulated, all the 22 field isolates of *Aeromonas hydrophila* used in this study revealed diversity in respect of the plasmid profile and antibiogram. The plasmid occurrence rate (POR) was 100 % among these isolates, as all the isolates carried at least one plasmid. Though, Bell and Trust (1989) has reported a POR of 100% in *Aeromonas salmonicida* isolates, the POR reported by many workers were of a lower order. As for example Chang and Bolton (1987) reported a POR of 38.7% in *A. sobria* and 19.5% in *A. hydrophila*. The plasmid occurrence rates in A. hydrophila isolates from several geographical locations were 14.7% (Ansari *et al.*, 1992) 46% (Borrego *et al.*, 1991). The presence of antibiotics advertently and inadvertently reaching the water bodies which are being used for aquaculture may be a factor leading to 100% POR in the *A. hydrophila* isolates observed in this study. It is to be expected as the presence of plasmids permit the bacteria to survive better in adverse environments, for example antibiotics (Luis *et al.*, 1998).

The plasmid profile of field isolates showed considerable diversity. The diversity was in respect of the number of plasmids harboured as well as their size. The plasmid number varied from 1 to 4 in isolates. While 50% of the isolates had single plasmid, 18% had two plasmids, 23% had three and 9% had four plasmids in them. Though the isolates could be classified into four groups on the basis of plasmid number, the plasmid profiles were not uniform in the groups with two or more plasmids. These groups showed diversity in respect of the molecular size of their plasmids DNA, and so could be classified into sub-groups on the basis of the molecular weights. The variation in molecular size has resulted in their differential migration in the electrophoretic gel leading to different banding patterns. All together 8 different plasmid DNA profiles were resolved from the 22 isolates through agarose gel electrophoresis. While all the isolates with single plasmid were uniform with respect to their molecular weight and so were of a single profile, the two isolates (Ah_1 and Ah_3) having four plasmids in them had different profile. While three different plasmid profiles were observed among the five isolates (Ah_2, Ah_5, Ah_{13}, Ah_{15} and Ah_{16}) with three plasmids each, only two plasmid profiles were present in the four isolates (Ah_4, Ah_{18}, Ah_{21} and Ah_{22}) with two plasmids. Son *et al.* (1997) have reported diversity in the plasmid profile of *A. hydrophila* isolated from Telapia of Kula Lumpur, Malaysia. He detected diversity among the seven isolates with respect to size and intensities of plasmid bands. Similarly diversity in plasmid carriage between *Aeromonads* of Atlantic coast and Pacific coast has been observed by Dalsgaard (1994) and Pederson *et al.* (1996) .

The most common profile among the isolates in the present study consisted of a single plasmid of 21Kb size. While this profile was exhibited by 50% of the isolates, the other profile were of lower order ranging from 4.5% to 13.6% only. The most common plasmid profile in *Aeromonas spp.* Isolated from Brown bull heads of Buffalo River, New York reported by

Pettibone *et al.* (1996) consisted of a single plasmid of 11.0 Kb with an incidence of 70% (in 7 out of 10 isolates).

The 21kb plasmid, found in the most common profile was present in other profiles also i.e. it was shared by all the 22 isolates and therefore can be considered characteristic of the of the *A. hydrophila* populations under investigation. The additional plasmids found in the other isolates with less common profile might have been acquired by them. Bacteria acquired plasmids to empower itself for surviving in adverse conditions. Concurrently with the sharing of this major plasmid of 21 Kb by all the isolates, resistances to seven antibiotics were also shared pointing towards the plasmid-mediated resistance against them. Mc. Nicol *et al.* (1980) reported that plasmid antibiotic resistance in *Aeromonas* is associated with large plasmids. The antibiotic resistances shared by all the isolates harbouring the 21 Kb plasmid were ampicillin, amoxycillin, cephalexin, bacitracin, penicillin-G, vancomycin and lincomycin. These are the antibiotics of common use, may be with exception of the last two.

There were 8 antibiotics against which differential resistance was shown by the isolates. They were amikacin, cefaclor, carbenicillin doxycillin, erythromycin, neomycin, norfloxacin and oxytetracycline. The isolates with single plasmid also have shown differential resistance to these antibiotics pointing to the chromosomal rather than plasmid mediation for them. If the genes for these resistance were on account of their plasmid a more uniform resistance pattern would have been exhibited by those isolates harbouring 21Kb plasmid. Chromosomal gene control of resistance to certain antibiotics in bacteria have been suggested by Prescott *et al.* (1990) and confirmed by the work of Barnes *et al.* (1991) and Schmidt *et al.* (2001). Again, most of the additional plasmids present in the isolates with the variant profiles are of small size, except a mega plasmid in two isolates. According to Mc. Nicol *et al.* (1980) antibiotic resistance is associated with large plasmids

and the small plasmids found in *Aeromonads* are classified as cryptic. Chang and Bolton (1987) and Chaudhury *et al.* (1996) reported that the plasmids that encoded resistance in *A. hydrophila* varied in size from 85.6 Kb to >150 Kb. Therefore, the role of the small plasmids in providing antibiotic resistance to the isolates harbouring them is doubtful. However, Son *et al.* (1997) reported that in *A. hydrophila*, antibiotic resistance is mediated by plasmids of variable sizes including smaller ones. Likewise, Pettibone *et al.* (1996) observed that though all the plasmids of *Aeromonas* strains isolated by them were small in size ranging between 1.5 Kb and 12 Kb, there was association between the plasmid content and antibiotic resistance. However, to establish whether the genetic control of the antibiotics with differential resistance is plasmid mediated, further studies like plasmid curing, conjugation experiments and transformation of *E. coli* with these plasmids are to be conducted.

Plasmid profiling can be used as a tool for genetic characterization and for differentiating between strains within the species. The sharing of a major plasmid (21 Kb) by all isolates of *A. hydrophila* could be used as an easy to use DNA marker, since plasmid profile can be resolved through simple agarose gel electrophoresis and visualized over U.V. light. This may serve as a useful marker with application in the species level identification. The plasmid profile diversity among the isolates concurrently with sharing of certain plasmids at the same time observed in this study points towards the possible use of the plasmid profile as a marker for species level identification as well as differentiating between isolates within the species, in conjunction with other methods of characterization. Prem Kumar *et al.* (1991) observed that plasmid profile is useful in differentiating *E. coli* when used in conjunction with other methods of characterization.

Bast *et al.* (1988) evaluated the plasmid profiles of *A. salmonicida* as epidemiological markers and considered that they were too uniform to be useful. However, in this study considerable diversity were

noticed in the plasmid profiles of the isolates. To be precise there were 8 different profiles. Further work is needed to elucidate the application of the plasmid profile diversity observed among the *A. hydrophila* isolates in epidemiology.

The RAPD profile generated through the random or arbitrary primed PCR amplification of DNA can bring to light the enormous amount of genetic variation that remain hidden in other types of molecular characterization. Hardys *et al.* (1992) have made a similar statement after the RAPD analysis of crab DNA. Radu *et al.* (2000) reported that RAPD profile generated using arbitrary primed PCR of *Salmonella entritidis* was more sensitive than plasmid profiling and antibiotic resistance pattern with respect to the individualization of the isolates. Hence amplification of plasmid DNA using decamer random primers was carried out in the present study to evaluate the heterogeneity of plasmid DNA. The PCR amplification produced a number of amplicans scorable as distinct bands on agarose gel electrophoresis.

The plasmid DNA fingerprint thus generated reveled considerable amount of polymorphism between the isolates by way of variation in size and number of amplicons. Each plasmid profile group produced a unique RAPD profile leading to absolute polymorphism. Comparison of individual amplicons reveled that while some of the major amplicons were species specific, shared by all samples, some were polymorphic. The overall polymorphism revealed by POPGENE analysis of RAPD data was 86.02%. The polymorphic RAPD based plasmid DNA fingerprint pattern reflects the intra-species extra chromosomal DNA heterogeneity .

The RAPD analysis of plasmid DNA revealed enhanced levels of plasmid DNA diversity that was not evident in simple plasmid profiling,

making it more discriminatory and better tool for evaluation of extrachromosomal DNA diversity and epidemiological studies.

The proteins are the expressions of the genetic code in DNA. Therefore, the protein profile comparison shall reveal the functional genetic diversity. Mc Lean *et al.* (1993) suggested that cellular protein profiles have wide applications in characterization, classification and identification of bacterial isolates. The cellular proteins from all the isolates were therefore resolved and through both NATIVE-PAGE as well as SDS-PAGE. The overall protein profile thus generated was not uniform for all isolates. However, comparison of the individual protein bands revealed species specific ones shared by all the isolates. This was true for both NATIVE-PAGE and SDS-PAGE profiles. The proteins bands of 48 KDa, 43 KDA and 18 KDa resolved by SDS-PAGE were species specific. The SDS profile in this study has brought to light sufficient variation between the isolates to be used as protein fingerprint. The protein profiles though not as discriminatory as RAPD, is also a useful tool for species identification as well as evaluation of intraspecies diversity.

Fish infected with bacteria harbouring plasmid mediated antibiotic resistance shall be a health hazard for both aquaculture and public health aspects, as these resistance could be transferred to other potential fish and human pathogen through the conjugation process. Fish infected with resistant *A. hydrophila* could serve as a reservoir of bacterial resistance. Pathak *et al.* (1993b) warn that antibiotic resistant *Aeromonads* should be of public health concern because of problems associated with control and treatment of infections. Since *A. hydrophila* is a potential pathogen of fish (Olivier *et al.*, 1981) and human (Khardori and Fainstein,1988) factors like drug resistance, which enhances the ability of these bacteria to survive, has public health significance and should be accessed (Pettibone *et al.*, 1996).

6. Summary

SUMMARY

Twenty-two isolates of *Aeromonas hydrophila* collected from fish, water and sediment samples of aquaculture systems and confirmed to be *A. hydrophila* on the basis of morphological and physiological tests were used for the study.

All the 22 isolates were screened for the plasmid carriage. The plasmid occurrence rate was 100%. Intraspecies plasmid profile diversity was evident. There were 8 different types of plasmid profiles. However a 21 Kb plasmid was found to be shared by all the isolates. The most common plasmid profile was single banded (21Kb).

All the 22 isolates were checked for their susceptibility against 20 antibiotics. All the isolates were resistant to seven antibiotics viz. ampicillin, amoxycillin, cephalexin, vancomycin, bacitrcin, penicillin-G and lincomycin. All were sensitive to only five viz. ofloxacin, nitrofurazone, gentamycin, chloramphenicol and pefloxacin. Differential resistance was shown by the isolates against 8 antibiotics.

Plasmid DNA of different plasmid profile groups were subjected to PCR amplification using four Operon decamer random primer viz. OPA-01 to OPA04. PCR amplification of plasmid DNA with these primers resulted in 93 amplicons, which were consistent and reproducible.

Primers OPA-01, OPA-03 and OPA-04 generated 8,1and 4 non-polymorphic amplicons, which could serve as species-specific markers. Rest of the amplicons was polymorphic and highly discriminatory between isolates. POPGENE analysis indicated 86.02% polymorphism. RAPD of

plasmid DNA appeared to be an ideal tool for evaluating the extrachromosomal DNA diversity.

Cellular protein profiles of all the isolates were developed by using NATIVE-PAGE as well as SDS-PAGE technique. The profiles were not uniform among the isolates, indicating intraspecies diversity.

A total of 12 different types of protein bands were resolved through NATIVE-PAGE, out of which six were shared by all. The remaining six protein bands showed differential sharing. Cellular protein analysis through SDS-PAGE technique produced a total of 14 protein bands. Three of them were shared by all isolates while 11 were differentially expressed. While those proteins expressed by all isolates could be considered for species identification, the differentially expressed ones could be used to evaluate the intraspecies diversity.

Though all the techniques used in this study indicated intraspecies heterogeneity in *A. hydrophila* from the aquaculture systems, the RAPD analysis of plasmid DNA revealed high level of diversity than others making it a better tool for evaluation of extrachromosomal diversity and epidemiological studies.

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